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RACEMIZATION OF THE PROTEINS OF *VIBRIO CHOLERÆ* AND RELATED ORGANISMS.

Part I.

THE DIAMINO ACIDS

BY

B N MITRA, M sc.

(Cholera Inquiry, Indian Research Fund Association, All-India Institute of Hygiene and Public Health, Calcutta)

KOSSEL (1912) and subsequently Dakin (1912) observed that proteins on treatment with alkali suffer a progressive loss in their optical activity

The amino acids obtained from proteins by acid hydrolysis are all, with the exception of glycine, optically active, but those derived from proteins which have previously been treated with dilute alkali for a time and then hydrolysed with acids are for the most part inactive This change is called racemization, an unsuitable term, since the loss of optical activity probably does not result from a mixture of dextro- and lævo-rotatory forms, but from a conversion into an inactive form Dakin and Dudley (1912, 1913) explained the reaction by assuming that under the influence of dilute alkali an intra-molecular change in the protein molecule took place similar to keto-enol tautomerism in the peptide groups —CH CO— The amino acids occupying the terminal positions of the peptide chains are not easily affected by dilute alkali, and therefore, on hydrolysis should appear in the normal, active form The racemization on the other hand occurs in those acids which are completely built into the molecular structure of the protein This has also been demonstrated by Levene, Steiger and Marker (1931), and Levene and Steiger (1932), working with polypeptides While possibly some hydrolysis of the protein also takes place along with the racemization, Levene and Pfaltz (1925) believed that under the influence of weak alkali racemization probably precedes hydrolysis

Investigations of proteins by the method of racemization have been very useful in detecting the structural differences of proteins which are apparently very similar in nature   Briefly, the method consists in treating the proteins with dilute alkali at 37°C for several days when the optical activity gradually falls to a constant value   The proteins are then said to have been racemized   They are subsequently hydrolysed with an acid and the optical activity of the individual amino acids which are liberated are compared   If in two related proteins the same amino acids are racemized it is inferred that they have the same relative positions

Thus by making a comparative study of the optical properties of amino acids released after hydrolysing the racemized caseinogens of the cow and the sheep, Dudley and Woodman (1915) could show that they were structurally different Similarly Dakin and Dale (1919) were also able to detect the differences in the chemical structure between the egg-albumins of the hen and of the duck

Woodman (1921) by a simplified process of racemization could establish the chemical identity of globulins of cow and ox sera and of colostrum, also that of milk-albumin and colostrum-albumin, and the non-identity of lactalbumin and serum-albumin   Woodman's method was a simplification in that it did not necessitate the actual isolation of amino acids from racemized proteins and the studies of their optical properties, but only required the plotting of the curve of specific rotation of the proteins against time under the influence of dilute alkali at 37°C   According to Woodman, all the proteins to be pronounced structurally the same must exhibit the same sort of curve

Elementary analyses failed to show any differences in the constitution of proteins of vibrios (Linton, Shrivastava and Mitra, 1934) , nor did the distribution of nitrogen by the method of van Slyke (Linton, Mitra and Shrivastava, 1934a) The amino acids of which the proteins are built up appeared from these studies to be quantitatively the same   The simplified method of racemization as developed by Woodman did show, however, that there are in the vibrio group at least two types of proteins which are structurally different, and we have designated them as protein I and protein II (Linton, Mitra and Shrivastava, 1934b) The vibrio proteins on treatment with alkali follow either of the two smooth curves of racemization Protein I, which is mostly derived from pathogenic vibrios, has an initial specific rotation of about —80° and gradually drops down to —19° in about 240 hours' incubation in N/2 alkaline solution, whereas protein II, which is mostly derived from non-pathogenic vibrios and from water vibrios, has an initial specific rotation of about —74° and a final specific rotation of —14° after about 240 hours' incubation in N/2 alkaline solution   This finding has been of significance in that it has led to a classification of cholera vibrios on the basis of the chemical structure (Linton, Shrivastava and Mitra, 1935), and since it may have a bearing on the serological relations of the vibrios and perhaps on their toxicity

It was of interest to find out what differences existed in the structures of protein I and protein II, and with this end in view the optical properties of the amino acids released after acid hydrolysis of the racemized material have been studied   I will confine myself in this part with the results on hexone bases and deal with those on other amino acids in the next part

METHOD OF PREPARATION OF VIBRIO PROTEINS.

The vibrios were sown on an agar medium of pH 7 8   The agar medium contained 5 per cent agar and papain-digest broth containing 1 per cent oxidizable matter   The heavy growth obtained after 48 hours' incubation at 37°C was washed off in 0 5 per cent phenol, centrifuged and washed repeatedly with distilled water in the Sharples Supercentrifuge till it was free from any agar which might have been carried over along with the bacterial mass   The washed product was then dried in an air-oven at 50°C   The usual yield of the protein from 100 bottles was about 15 grammes

EXPERIMENTAL

Sixty grammes each of vibrio proteins I and II were separately treated with 600 c c of N/2 NaOH and kept in the incubator at 37°C and tested at intervals for 12 days, when the optical rotation had dropped down to a constant value in each case   The solution was then centrifuged and the clear fluid neutralized with sulphuric acid and then treated with glacial acetic acid till the racemized protein separated out   The protein was centrifuged off, washed free from acid and dried

Weight of racemized protein I=14 2 g (23 67 per cent), and weight of racemized protein II=15 3 g (25 50 per cent)

Each sample of racemized protein was treated with 100 c c of 30 per cent H_2SO_4, and hydrolysed on a sand-bath for about six hours till the biuret test on a few drops of the hydrolysate was negative   The sulphuric acid was then quantitatively removed with barium hydroxide and the solution was decolorized with animal charcoal and filtered   The filtrate and washings were concentrated *in vacuo* at 60°C to less than half the original volume

The method for the separation of the diamino acids was essentially that of Kossel and Kutcher (1899) as described by Plimmer (1917)   It consisted in the precipitation of arginine and histidine as their silver salts and their separation by difference in solubility in water and in strong alkaline solution, and the precipitation of lysine from the filtrate by means of phosphotungstic acid after the silver compounds had been removed   These in turn were isolated as arginine nitrate $C_6H_{14}N_4O_2 HNO_3 \frac{1}{2}H_2O$, histidine hydrochloride $C_6H_9N_3O_2 2HCl$ and lysine picrate $C_6H_{14}N_2O_2 C_6H_2(NO_2)_3OH$ The free bases of arginine and histidine were then prepared from the purified compounds by treating with the requisite amount of alkali and then concentrating the solution *in vacuo* until crystals began to appear   After standing for a day or two the crystals were centrifuged off washed with cold alcohol and dried *in vacuo* over soda-lime.   Free lysine was prepared from its chloride obtained by decomposing the picrate   Lysine chloride was then treated with an excess of Ag_2O in a slightly acid solution, the silver in its turn being removed by H_2S, and the acid exactly by $Ba(OH)_2$ Crystals of lysine began to appear on concentration *in vacuo* and thereafter were purified with as little exposure to the air as possible by washing with ice cold water, and then dried *in vacuo* over soda-lime (Vickery and Leavenworth, 1928)

RESULTS

The diamino acids were prepared from both racemized protein I and protein II, and their properties are illustrated in the following tables —

TABLE I

Amino Acids	Protein number	Per cent yield	Melting point °C	Known m p (Vickery and Leavenworth, 1928)	Per cent N	Per cent N theoretical
Arginine (anhydrous)	1	4 2	230–35	238 (decomp)	32 00	32 18
	II	3 8	228–34		31 98	
Histidine	I	1 7	268–70	277	27 28	27 10
	II	1 8	268–72	.	26 92	
Lysine	I	3 0	210–20	224 (decomp)	18 96	19 17
	II	3 3	212–20		19 00	

The diamino acids were obtained from the two types of proteins in almost equal quantities and their respective nitrogen content and melting point showed that they were quite pure

TABLE II

Specific rotations of the amino acids in aqueous solution   Temperature 30°C

Amino Acids	Protein I	Protein II	Known
Arginine	+ 6 4° (2 02 per cent)	0 0 (1 80 per cent)	+ 12 5° (Vickery and Leavenworth, 1927)
Histidine	0 0 (1 02 per cent)	0 0 (0 90 per cent)	— 7 59° (Kossel and Kutcher, 1899)
Lysine	+ 13 3° (1 81 per cent)	+ 13 5° (1 85 per cent)	+ 14 6° (Vickery and Leavenworth, 1928)

Histidine is optically inactive in both the racemized proteins, while lysine preserves its optical activity in both the cases   Arginine on the other hand is partially active in protein I and totally inactive in protein II

These diamino acids were also prepared from unracemized protein I for the sake of comparison, that is to say, the protein without first being treated with alkali was hydrolysed with the requisite amount of strong sulphuric acid and the

subsequent process for the isolation and purification of these acids were in every way the same   The following were the results obtained from 40 g of unracemized protein I —

Arginine—Yield=1 60 g (4 0 per cent) , m p = 228°C to 232°C , N = 32 10

per cent , $\left[\alpha \right]_D^{30} = + 12\ 0°$ (4 per cent aq)

Histidine—Yield=0 60 g (1 5 per cent) , m p = 268°C to 272°C , N = 26 99

per cent , $\left[\alpha \right]_D^{30} = - 7\ 5°$ (2 per cent aq)

Lysine—Yield=1 10 g (2 75 per cent) , m p = 215°C to 220°C , N = 19 0

per cent , $\left[\alpha \right]_D^{30} = + 14\ 0°$ (4 per cent aq)

It is evident from these findings that the method used gives results entirely concordant with those of other workers for unaltered amino acids

Discussion

From the above results it is clear that in both the types of proteins histidine and lysine behave in the same way   On treatment of the proteins with dilute alkali for a long period, histidine is totally racemized in both the cases, whereas lysine remains unaffected   This shows that in both cases lysine occupies a terminal position in the chain, while histidine occurs within the structure of the molecule   Arginine, however, is found to behave quite differently   In protein I it is partially racemized, and in protein II it is fully racemized   It follows, therefore, that the position of arginine with respect to other amino acids in the protein molecule differs in protein I and protein II since it is acted upon differently by the alkali   I may conclude that structural differences do exist between protein I and protein II with respect to the configuration of arginine in the two molecules

Summary

1   The diamino acids have been isolated from racemized protein I and racemized protein II of cholera vibrios, and their optical properties studied.

2   A difference in the configuration of arginine within the molecule of the two types of proteins of cholera vibrios has been demonstrated

3   The relative positions of both histidine and lysine in the two protein molecules are the same

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RACEMIZATION OF THE PROTEINS OF *VIBRIO CHOLERÆ* AND RELATED ORGANISMS

Part II.

THE MONOAMINO ACIDS

BY

B N MITRA, M sc

(*Cholera Inquiry, Indian Research Fund Association, All-India Institute of Hygiene and Public Health, Calcutta*)

THE first part of this paper (Mitra, 1936) dealt with the isolation of the individual hexone bases from the racemized protein I and protein II of cholera vibrios and it was shown from a study of the optical behaviour that the position of arginine in the molecule of the two types of proteins differs   In this part will be presented the results of the investigations on other amino acids obtained from the racemized proteins of the two types

RACEMIZATION

Four hundred grammes each of protein I and protein II, prepared in the same way as described in the previous part were finely powdered taken up separately in 4 litres of N/2 sodium hydroxide solution and allowed to stand in the incubator for 12 days, when the optical rotation of the solution was found to have fallen to a constant value   They were then taken out and centrifuged in the Sharples Supercentrifuge and the dark-brown liquid portion containing the racemized protein was rendered neutral with sulphuric acid, and glacial acetic acid added to maximum precipitation   The precipitate was centrifuged off, washed with water, rendered neutral to litmus with caustic soda, dried in the air-oven at 50°C and weighed

The weight of recovered protein I was 104 g , and that of protein II 98 g , that is to say, nearly 25 per cent of the whole protein was recovered in a racemized condition

EXPERIMENTAL

Hydrolysis —The two racemized proteins were hydrolysed by boiling with 600 c c of 30 per cent sulphuric acid on a sand-bath for 6 hours when the biuret test on a few drops of the hydrolysate was negative

Treatment of the hydrolysate —The hydrolysate was then further diluted with water, warmed, and the sulphuric acid quantitatively removed with barium hydroxide   The hydrolysate which was still faintly acid to litmus was decolorized with charcoal and filtered by suction   The barium sulphate precipitate was repeatedly washed with hot water till a test drop no longer gave any Millon's test for tyrosine and the washings added to the filtrate   The filtrate containing the amino acids was then concentrated at 60°C *in vacuo* until the tyrosine began to separate out   It was then allowed to stand overnight in the ice-box   The precipitate containing mostly tyrosine and also some leucine was then filtered off and the mother liquor further concentrated till no more tyrosine appeared and the mother liquor did not respond to Millon's test   The examination of this precipitate, the weight of which after washing with alcohol and ether and drying at 105°C was 7 8 g , will be dealt with later

Extraction of amino acids with butyl alcohol —Dakin (1918, 1919, 1920) found that by long-continued extraction of a protein hydrolysate with isobutyl alcohol under reduced pressure, he was able greatly to increase the summation of amino acids, and the amino acids so obtained were easily separable and could be crystallized in the free state   This process is unique in that it causes no racemization   Briefly, the butyl alcohol method separates the constituents of the hydrolysed product into the following main groups —

1   Solid monoamino acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol

2   Proline, soluble in butyl alcohol and extracted by it   (Some diketopiperazines may also be present the formation of which is greatly minimized by carrying out the extraction under reduced pressure)

3   The dicarboxylic acids as also the diamino acids, not extracted by butyl alcohol, the latter being subsequently removed by phosphotungstic acid in sulphuric acid (5 per cent) solution   This fraction may also contain some glycine and possibly hydroxyproline

The amino acids derived from racemized protein I and protein II were extracted by butyl alcohol according to Dakin's method   The very thick mother liquor, left over after the separation of tyrosine together with some leucine, was placed in a continuous liquid extractor with 500 c c isobutyl alcohol at 60°C to 65°C under a reduced pressure (about 12 mm) maintained by an efficient water pump   The extraction was continued for 48 hours day and night   Soon after extraction began, a granular precipitate began to appear on the sides of the extraction vessel, and after 24 hours' extraction, the precipitate in the extraction flask was removed and the extraction continued with the same amount of fresh butyl alcohol   A very small quantity of precipitate was obtained during the next 24 hours and the extraction was then discontinued

The butyl alcohol was removed from the precipitate by filtration after standing in the ice-box for some time, and was concentrated *in vacuo* to a very small volume,

allowed to stand in the ice-box and the further precipitate which appeared was recovered by filtration. The precipitates were added together, washed with alcohol and ether, and dried at 105°C. The weight was 28 4 grammes, its nitrogen content 12 25 per cent, and specific rotation of 4 per cent aqueous solution at 30°C was —12 5° This portion contained mostly leucine, phenylalanine, alanine, valine and hydroxyproline. The figures given here together with those that follow were derived from racemized protein I.

Examination of solid amino acids —LEUCINE —The solid amino acids were then taken up in water and the solution was highly concentrated *in vacuo* and left in the ice-box for 2 days. Leucine and phenylalanine being sparingly soluble were the first to crystallize out and by repeating the process they were almost quantitatively removed from the other acids by filtration. The precipitate of leucine and phenylalanine was then washed with ice-cold alcohol (95 per cent), and recrystallized from water and dried. The weight of the precipitate was 11 0 grammes and the nitrogen content of the mixture=10 6 per cent

Leucine has 10 69 per cent N ⎫
Phenylalanine has 8 49 per cent N ⎭ difference = 2 2

Hence percentage of leucine in the mixture $= \dfrac{10\,60 - 8\,49}{2\,2} \times 100 = 95\,9$ per cent

The amount of phenylalanine in the mixture was very small, if indeed it was actually present

Leucine was purified by repeated crystallization from water. The quantity recovered was 9 95 g, m p = 290°C to 293°C, N = 10 56 per cent, $\left[\alpha\right]_D^{30} = 0\,0$ (2 per cent aq)

A further crop of leucine was also obtained from the mixture of tyrosine which was collected after the concentration of the protein hydrolysate prior to the extraction of amino acids with butyl alcohol and will be described later

GLYCINE —The mother liquor after the separation of leucine and phenylalanine was esterified by Fischer's esterification process (Fischer, 1901), concentrated at 40°C under pressure, and left overnight in the ice-box. The glycine ester hydrochloride separated out because of its insolubility and was filtered off. The filtrate was again saturated with hydrochloric acid, and concentrated, and a further crop of glycine ester hydrochloride was obtained. The glycine ester was liberated from its hydrochloride by the barium hydroxide method of Levene and van Slyke (1909), decolorized, and distilled below 60°C at 10 mm to 12 mm pressure and finally hydrolysed by boiling with water. The glycine was purified by crystallizing from water

Weight = 3 8 g, m p = 218°C to 220°C, N = 18 50 per cent

A third crop of glycine was recovered from the aqueous layer left over after the extraction with butyl alcohol

It should be noted here that the esters were always liberated from their hydrochlorides by barium hydrate in a solution which had been concentrated *in vacuo* at 40°C as proposed by Levene and van Slyke (*loc cit*) This method for liberation of ester was the only one employed. Barium hydrate has the advantage over a stronger alkali that it causes no saponification and can be easily removed

ALANINE —The mother liquor after the separation of glycine ester hydrochloride was treated with baryta to free the ester from hydrogen chloride and fractionally distilled at a pressure of about 10 mm to 12 mm Alanine distilled below 60°C and was hydrolysed and carefully purified by crystallizing from water since it might carry with it traces of glycine and also leucine as impurities

Weight = 6 65 g , m p = 292°C to 296°C , N = 15 56 per cent $[α]_D^{30} = 0\ 0$ (4 per cent aq)

VALINE —The fraction boiling between 60°C and 90°C was collected and hydrolysed The probable impurities in this fraction were traces of leucine, isoleucine and alanine In order to remove these, the valine solution was rendered alkaline with ammonia, a few drops of dilute lead acetate solution added and the precipitate of lead salt of any leucine and isoleucine that might be present was filtered off (Levene and van Slyke, *loc cit*), but neither could be detected The filtrate was freed from lead by means of sulphuretted hydrogen and the solution after filtration evaporated to dryness Valine was further purified by treating the residue first with alcohol-ether mixture (3 1) and then recrystallizing it from water

Weight = 3 2 g , m p = 318°C to 320°C , N = 11 62 per cent , $[α]_D^{30} = 0\ 0$ (4 per cent aq)

The figure for the percentage of nitrogen of valine thus obtained was sufficient to show that the material was fairly pure, and did not therefore require any further fractionation

HYDROXYPROLINE —The residual ester left after distillation of other esters to about 90°C was extracted by ether, the ether removed by distillation, and the remaining material was then hydrolysed with water, and concentrated under pressure The hydroxyproline which separated out was purified by crystallization from 90 per cent methyl alcohol

Weight = 0 92 g , m p = 260°C to 262°C , N = 10 48 per cent , $[α]_D^{30} = -70°$ (1 per cent aq)

A further crop of hydroxyproline was also isolated by a similar method from the aqueous residue remaining from the butyl alcohol extraction

Hydroxyproline might be contaminated with serine, which was looked for in the mother liquor from the hydroxyproline but could not be detected

TYROSINE —As we have shown above, this amino acid was isolated on concentrating the protein hydrolysate on a water-bath to a thick semi-crystalline mass prior to the extraction of other amino acids by butyl alcohol, and was contaminated with leucine The weight of the dried material so obtained was 7 8 g and per cent N=8 72 Tyrosine was recovered from the mixture by treating it with glacial acetic acid when leucine passed into solution (Habermann and Ehrenfeld, 1902) Tyrosine was further purified by rendering it alkaline with ammonia and then treating it with glacial acetic acid so that the solution was still alkaline Tyrosine precipitated out and was filtered off It was finally washed with hot water several times (Abderhalden and Pregl 1905) The mother liquor was then acidified to precipitate out cystine, if present, but no yield was obtained

Weight $= 5\,14\,g$, m p $= 310°C$ to $316°C$, N $= 7\,68$ per cent , $[α]_D^{30} = 0\,0$ (4 per cent in 20 per cent HCl)

Leucine recovered from the mixture of tyrosine was purified by crystallizing from water

Weight $= 1\,4$ grammes

This weight together with the previous weight of leucine brought it to a total of 12 35 g so that the percentage of leucine was 10 9

Examination of butyl alcohol solution —PROLINE —Most of this remained in solution in the butyl alcohol after the separation of solid amino acids The alcoholic solution was further evaporated *in vacuo* to a syrupy-like consistency, redissolved in alcohol, allowed to stand for two days in the ice-box when a very small quantity of solid substances, possibly other amino acids, separated out and were filtered off The filtrate was further concentrated to a very small volume (25 c c) when all the alcohol had been evaporated off, and was then taken up in water and further purified by treatment with saturated mercuric acetate solution and barium hydroxide (Dakin, 1920) Only a very small precipitation of other amino acids occurred and the proline was left behind in aqueous solution The solution was then freed from barium by treatment with sulphuric acid and from the mercuric salt by saturating with sulphuretted hydrogen in a closed vessel After filtration, it was treated with charcoal, filtered again evaporated to dryness *in vacuo*, further purified by treating it with alcohol and then evaporating the alcoholic extract to dryness *in vacuo*

Weight $= 3\,4$ g , m p $= 218°C$ to $220°C$, N $= 11\,92$ per cent , $[α]_D^{30} = -\,62\,0°$ (4 per cent aq)

The proline thus obtained was a mixture of active and racemic forms The l-proline has a specific rotation of $-77\,4°$ (Dakin, 1920) Therefore, the percentage of active variety in the mixture $= \frac{62\,0}{77\,4} \times 100 = 80\,4$ per cent

Similarly, the percentage of active variety of proline derived from racemized protein II was 38 77, calculated on the basis of the specific rotation which was $-30°$

Examination of the mother liquor left after the butyl alcohol extraction —The mother liquor remaining after the extractions already described contained mostly the hexone bases, dicarboxylic acids and some glycine and hydroxyproline These constituents were separated by the method of Dakin (1920) The bases were removed by precipitation with phosphotungstic acid in 5 per cent sulphuric acid solution The phosphotungstic acid precipitate was not worked with, as this had already been done and is reported in the previous paper The filtrate was then treated with an excess of barium hydroxide and filtered The filtrate now contained the barium salts of dibasic acids, glycine and hydroxyproline, and was concentrated *in vacuo* to a very small volume, treated with ten volumes of alcohol, and filtered The precipitate was taken up in a small quantity of water and barium removed with just the requisite quantity of sulphuric acid, concentrated and left at about $-10°C$ for 5 or 6 days when considerable quantities of both aspartic and glutamic acids crystallized out, and were filtered off, washed and dried

The weight of the mixture of aspartic and glutamic acids was 17 3 g and its nitrogen content 9 72 per cent

Aspartic acid has 10 53 per cent N
Glutamic acid has 9 50 per cent N $\Big\}$ difference = 1 03

Hence percentage of aspartic acid in the mixture $= \frac{9\ 72 - 9\ 50}{1\ 03} \times 100 = 21\ 3$, that is, about 3 2 g of aspartic acid and 14 1 g of glutamic acid were present

The alcoholic mother liquor of these acids was also exactly freed from barium hydroxide with sulphuric acid and added to the mother liquor obtained after the separation of the dibasic acids, concentrated *in vacuo,* and finally esterified On concentration, glycine ester hydrochloride precipitated out Hydroxyproline was recovered from higher boiling fraction by extracting with ether as described before Weight of glycine recovered=1 2 g , and weight of hydroxyproline=1 3 g These weights together with those of glycine and hydroxyproline found previously bring the weights of glycine and hydroxyproline to 5 0 g and 2 22 g and the percentages to 4 8 per cent and 2 10 per cent, respectively

The residual fraction left after extraction with ether was hydrolysed and on concentration gave a further yield of 0 22 g with a nitrogen content of 9 3 per cent and proved to be a mixture of aspartic and glutamic acids

GLUTAMIC ACID —This acid was separated from the mixture of the aspartic and glutamic acids as the hydrochloride (Hlasiwetz and Habermann, 1873) which was formed by saturating the concentrated solution with dry gaseous hydrogen chloride The glutamic acid hydrochloride separated out on standing in the ice-box for some days A further crop was similarly obtained by again treating the mother liquor with gaseous hydrogen chloride The acid was obtained in its free state by decomposing the hydrochloride with ammonia, evaporating it to dryness, and crystallizing from water

Weight = 13 2 g , m p = 200°C , N = 9 40 per cent, $[\alpha]_D^{30} = +\ 9\ 5$ (4 per cent aq)

ASPARTIC ACID —After removing the glutamic acid hydrochloride the mother liquor was evaporated almost to dryness *in vacuo,* the residue was dissolved in water and boiled with just sufficient lead oxide to arrest all the chlorine It was then filtered, the lead removed from the filtrate by means of sulphuretted hydrogen, filtered again and concentrated *in vacuo* Aspartic acid crystallized out

Weight = 2 0 g , m p = 246°C to 248°C , N = 10 36 per cent, $[\alpha]_D^{30} = 0\ 0$ (2 per cent aq)

TRYPTOPHANE —Sulphuric acid completely destroys tryptophane and, to obtain it in an unaltered condition, the proteins are generally digested with active pancreas extract As the isolation of tryptophane requires a large quantity of the protein it was not attempted

RESULTS

The isolation and purification of the amino acids from racemized protein II were carried out exactly in the same way as in protein I and identical amino acids with closely similar percentages were obtained in both cases The findings in the

B. N. Mitra

two types of proteins are given in Table I, and the differences in their optical behaviour are shown in Table II —

TABLE I

Amino acids	Protein number	Per cent yield	Melting point °C	Known m p °C	Per cent N	Per cent N theoretical
Glycine	I	4 8	218–220	225–30	18 50	18 67
	II	4 0	217–221		18 54	
Alanine	I	6 4	292–296	297	15 56	15 73
	II	5 1	296–298		15 62	
Valine	I	3 0	318–320	315	11 62	11 96
	II	3 4	318–320		11 48	
Leucine	I	10 5	290–29?	293–95	10 56	10 69
	II	11 2	290–295		10 52	
Isoleucine	I	Not found				
	II					
Phenylalanine	I	Not found				
	II					
Tyrosine	I	4 9	310–315	314–18	7 68	7 74
	II	4 0	312–315		7 82	
Serine	I	Not found				
	II					
Cystine	I	Not found				
	II					
Aspartic acid	I	1 9	246–248	251	10 36	10 53
	II	2 4	244–246		10 45	
Glutamic acid	I	12 7	200	197–98	9 40	9 50
	II	13 8	200–202		9 42	
Proline	I	3 3	214–218	220–22	11 92	12 17
	II	3 8	214–220		11 88	
Hydroxyproline	I	2 1	260–262	270	10 48	10 69
	II	1 5	260–262		10 50	

Table I shows that the percentage yields of the amino acids were the same in both the types of proteins The melting points closely approximated those of known amino acids showing that they were obtained in a pure state The same was demonstrated by their respective nitrogen contents The four amino acids not found were possibly present in too small a quantity for isolation

TABLE II

Specific rotations of amino acids derived from racemized proteins of cholera vibrios in 4 per cent aqueous solution Temperature—30°C

Amino acids	Protein I	Protein II	Known (Mitchell and Hamilton, 1929)
Glycine			
Alanine	0 0	0 0	+ 2 7
Valine	0 0	0 0	+ 6 42
Leucine	0 0	−8 5	−10 35
Tyrosine	0 0	0 0	− 8 07*
Aspartic acid	0 0	0 0	+ 3 8
Glutamic acid	+ 9 5	0 0	+10 3
Proline	−62 0	−39 0	−77 4
Hydroxyproline	−70 0	−72 0	−81 04

* 4 per cent in 21 per cent HCl

Glycine is as usual optically inactive The other amino acids which were found optically inactive in both the racemized proteins are alanine, valine, tyrosine and aspartic acid, all of which, according to the theory are completely built into the structure of the protein molecules In protein I leucine is inactive and so occurs within the molecule , but in protein II it is optically active showing that it occupies the terminal position of a peptide chain in the molecule Glutamic acid on the other hand is optically active only in protein I, so that it is at the end of the peptide chain in protein I molecule but within the protein II molecule Proline is partially active in protein I and less active in protein II This indicates that the proline is

differently distributed in the two proteins   Hydroxyproline is, however, optically active in both the cases and thus occupies the terminal positions of the peptide chains in both the proteins

<div align="center">TABLE III</div>

A comparative table showing the optically active substances derived from both the types of racemized proteins of cholera vibrios and which are as such supposed to be at the end chains of the molecules

Protein I	Protein II
Glutamic acid	Leucine
Some proline (80 4 per cent)	Less percentage of proline (38 77 per cent)
Hydroxyproline	Hydroxyproline

The structural differences in the molecules of the two types of vibrio proteins are thus considerable and probably preclude any transmutation of a vibrio with protein I to one with protein II

The differences in the serological reactions of vibrios may be explained in part by the difference in the chemical structure that exists in the two types of proteins of vibrios and in part by differences in specific polysaccharides

<div align="center">SUMMARY</div>

1   A comparative study of the chemical structure of the two types of vibrio proteins with respect to the relative positions of the amino acids was made by the method of racemization

2   The isolation of the individual amino acids from the racemized proteins of cholera vibrios was greatly simplified by extracting them with isobutyl alcohol under reduced pressure (Dakin) and suitably supplementing the method by Fischer s esterification process

3   The extraction of glycine and hydroxyproline by butyl alcohol seemed to be incomplete

4   Glycine, alanine valine, leucine tyrosine, aspartic acid glutamic acid, proline and hydroxyproline were all obtained in approximately the same quantities from racemized proteins of the two types of vibrio proteins and their optical properties compared

5   The differences in the configuration of glutamic acid and leucine in the two protein molecules were established   These amino acids were found to differ completely in their relative positions   Proline, too, was found to be differently distributed in the two molecules, since more of it was racemized by alkali in protein II than in protein I

ACKNOWLEDGMENT

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RESPIRATION AND GLYCOLYSIS OF THE CHOLERA AND CHOLERA-LIKE VIBRIOS

BY

RICHARD W LINTON, PH D,

B N MITRA, M SC,

AND

D N MULLICK, M SC

(Cholera Inquiry, Indian Research Fund Association, All-India Institute of Hygiene and Public Health, Calcutta)

WE are reporting here the results of a study of the respiration, and the aerobic and anaerobic glycolysis, of a series of sixty-seven vibrios from various sources Most of them have also been classified from the point of view of their chemical structure, and their metabolism has been considered in relation to these groupings

METHOD

The organisms were grown on agar at pH 7 8 for 18 hours, and taken up in Ringer's solution   In the respiration experiments the Ringer's solution contained 0 1 per cent glucose, and in the glycolysis experiments 0 1 per cent glucose and 0 1 per cent $NaHCO_3$ The amount of oxygen consumed in the absence of glucose was found to be negligibly small

The experiments were carried out in a Barcroft's differential manometer at 37°C   and 0 5 c c of bacterial emulsion was used in each case   The weight of the bacteria in the emulsion was determined by drying a sample to constant weight at 50°C and weighing   The readings were taken after the experiment had gone on for 40 minutes   For aerobic glycolysis, the observations were made in the presence of air and for anaerobic glycolysis in the presence of nitrogen

RESULTS

The results are presented in the tables   Respiration is given in c mm of oxygen consumed per mg of bacteria, and glycolysis in c mm CO_2 given off per mg of bacteria   The strains are arranged in the tables according to the

chemical groups to which they belong, except in the case of four strains not yet classified completely, seven strains received from the Field Inquiry, and five strains of the ' medusa-head ' colony type, which differ from the other strains in respect to the vanishingly small amounts of glucose they use anaerobically

Respiration —The respiration of the group I strains is consistently high and without much variation from one strain to another   The figures for groups II and III overlap, but in general the respiration of the water vibrios is lower than that of the group II vibrios, which with the exception of vibrio ' Tank 65 (*b*) ' are directly from human sources   Strain ' Tank 65 (*b*) ' was isolated from a tank liable to contamination in a village where cholera was present

In groups IV, V and VI, respiration is found to be as high as in group I, although with a wider range between maximum and minimum   In the ' medusa-head   group it is, on the other hand, definitely low and only about one-fourth the amount found in the group I strains

Aerobic glycolysis —The findings in respect to aerobic glycolysis are of considerable interest   The first three chemical groups show a steadily decreasing power to utilize glucose in the presence of oxygen and in group IV the ability is completely lacking   The finding is true not only for the nine El Tor strains studied, but also for the strains of the same structure which have been found and identified chemically in India

In groups V and VI the aerobic glycolysis increases and in the latter is of the same order as in group I   In the ' medusa-head ' group, this activity, as in the case of respiration, is at a minimum, three of the strains being totally inactive, and the other two showing only slight utilization

Anaerobic glycolysis —No differences in this aspect of metabolism were found within the six cholera groups or in the unclassified and carrier strains   On the other hand, the ' medusa-head ' organisms use much less glucose, and the figures for anaerobic glycolysis were only about 10 per cent of those of the other groups

Unclassified group —These vibrios have not yet had a complete chemical analysis   One of them (vibrio 454) has already been found (Linton and Shrivastava, 1933) to possess the galactose-containing carbohydrate   It therefore falls either into group I or group IV   It was derived from a case of cholera, and the figures for its metabolism indicate a position for it in group I, as it does consume glucose aerobically, which the group IV organisms do not do   The other three strains were isolated from water, and the only chemical information yet obtained about them is that strain W25 has the arabinose-containing carbohydrate which is also found in the other water vibrios of group III   The metabolism of these three strains is of the same order as that of the vibrios having protein II and carbohydrate II, their averages in respiration, and in aerobic and anaerobic glycolysis being 0 54, 0 29 and 2 08 c mm respectively, while the corresponding figures for group III are 0 59, 0 28, and 2 07 c mm , thus showing a close correspondence

Mixed carbohydrate group —This group contains the strains in which more than one type of specific carbohydrate has been found (Linton, Shrivastava and Mitra, 1935)   The respiration and anaerobic glycolysis of these strains is high, and the aerobic glycolysis is more variable than that of the first six groups, principally on account of two Basrah strains, which are low in this respect   With these exceptions the other strains are as active as the group I vibrios

TABLE I

Metabolism in cholera and cholera-like vibrios

Strain	Respiration c mm O_2	Aerobic	Anaerobic	Strain	Respiration c mm O_2	Aerobic	Anaerobic
		GLYCOLYSIS				GLYCOLYSIS	
		c mm CO_2				c mm CO_2	
GROUP I				GROUP II			
1612	1 35	0 83	2 19	505	0 72	0 55	2 10
79 A	1 21	0 69	2 22	E	0 68	0 48	2 01
Rangoon Smooth	1 34	0 65	2 13	2027	0 76	0 50	2 08
Inaba	1 26	0 84	2 21	Tank 65 (*b*)	0 69	0 42	2 40
10404	1 20	0 60	2 15	1331 (agg)	0 72	0 58	2 11
1617	1 24	0 72	2 19				
Rangoon Rough (1)*	0 83	0 47	1 81				
GROUP III				GROUP IV			
W880	0 48	0 30	2 12	El Tor	1 24	0 0	1 93
W3075	0 68	0 32	2 08	El Tor I	1 31	0 0	1 90
10405	0 52	0 19	2 14	El Tor II	1 01	0 0	2 04
158	0 70	0 28		El Tor III	1 14	0 0	2 04
2092	0 50	0 29	1 96	El Tor IV	1 02	0 0	2 07
1331 (non agg)	0 69	0 30	2 00	El Tor V	1 25	0 0	1 94
				El Tor VI	1 09	0 0	2 04
				El Tor VII	1 33	0 0	2 04
				El Tor VIII	1 08	0 0	1 95
				79 B	1 16	0 0	2 15
				9320	1 14	0 0	2 30
				676	1 10	0 0	1 80

* See text

TABLE I—*concld*

Strain	Respiration c mm O_2	GLYCOLYSIS Aerobic c mm CO_2	GLYCOLYSIS Anaerobic c mm CO_2	Strain	Respiration c mm O_2	GLYCOLYSIS Aerobic c mm CO_2	GLYCOLYSIS Anaerobic c mm CO_2
GROUP V				GROUP VI			
Basrah II	1 16	0 29	1 95	China 5	0 80	0 50	2 04
Kohat Original	0 97	0 43	2 50	China 14	0 97	0 83	1 78
Kohat Current	1 03	0 47	2 30	6380 (agg)	0 98	0 82	2 10
Kohat Calcutta	0 92	0 41	2 10	1200	1 10	0 60	2 05
6380 (non agg)	1 30	0 40	2 00	1200	1 20	0 90	2 03
				Rangoon Recovered	0 97	0 83	1 99
				10405 (agg)	1 20	0 71	2 06
MIXED CARBOHYDRATE GROUP				UNCLASSIFIED GROUP			
Basrah I	1 50	0 46	2 03	454	1 15	0 65	2 04
Basrah III	1 11	0 20	2 13	W467	0 48	0 25	2 00
Basrah IV	0 92	0 20	2 13	W785	0 62	0 35	2 11
China 21	1 20	0 60	1 96	W25	0 53	0 27	2 13
China 22	1 00	0 70	2 00				
China 23	1 15	0 70	2 20				
1065	1 14	0 73	.				
W880 (agg)	0 96	0 84	2 10				
10405 T	1 00	0 70	2 40				
CARRIER STRAINS				'MEDUSA HEAD STRAINS			
390	0 83	0 28	1 97	Rangoon Rough (2)	0 30	0 0	0 0
952	0 80	0 60	2 06	El Tor I R	0 54	0 17	0 27
4951	1 10	0 50	1 97	El Tor II R	0 50	0 0	0 33
1312	1 05	0 55		China 22 D	0 32	0 0	0 38
950	0 80	0 42	1 96	10405 D	0 58	0 14	0 26
683	1 04	0 26					
467 .	0 88	0 72	2 17				

The carrier strains —These field strains have not yet been grouped chemically In metabolism they resemble the vibrios of groups II and V more closely than they do the others, a finding which may prove of epidemiological significance

Table II, which contains the averages of metabolic activities in the various groups, illustrates the statements made above —

TABLE II

Averages of results of metabolism of the six chemical groups, and the mixed, carrier, and 'medusa-head' groups

Group	Respiration c mm O_2	Aerobic glycolysis c mm CO_2	Anaerobic glycolysis c mm CO_2
I	1 27	0 72	2 18
II	0 71	0 49	2 14
III	0 59	0 28	2 07
IV	1 16	0 0	2 01
V	1 04	0 40	2 21
VI	1 01	0 73	1 96
Mixed	1 06	0 58	2 30
Carrier	0 92	0 47	2 06
'Medusa head'	0 45	*	0 25

* Three strains negative, two strains 0 14 and 0 17 c mm respectively

The metabolism of original and variant strains

In Table III are included the results of the metabolic study of a number of strains which have undergone variation in the direction of greater smoothness or roughness compared to their parent strains and have also shown changes in chemical structure Also included are the China strains and a number of agglutinable and inagglutinable strains from the same human sources in the field investigation All of these strains will be considered chemically in a future communication of our studies on the antigenic structure of the vibrios, Part X

The Rangoon strains (Linton, Shrivastava and Mitra, *loc cit*) —Rangoon Smooth has the metabolic powers associated with the group I strains   The rough form, Rangoon Rough (1), which has the same chemical structure except that it possesses a smaller amount of specific carbohydrate, is definitely lower in its respiration and glycolytic power in air   The third member of the group, Rangoon Rough (2), which is the first 'medusa-head' strain we encountered, and which differs completely from its parent Rangoon Rough (1) in chemical structure, is almost inert metabolically, as both types of glycolysis are absent and its respiratory exchange is at a minimum

The next strain, Rangoon Rough (2a), which represents a step in the derivation of a smooth vibrio agglutinating with Rangoon Smooth antiserum, from Rangoon Rough (2), shows that its intermediate colony, biochemical and serological characteristics are paralleled by its metabolism   In contrast to its parent, its respiration is twice as high, and it has fully regained its power of anaerobic glycolysis   Aerobic glycolysis is, however, still absent, and in this respect, as in the others mentioned, it is an intermediate strain

In the last strain of the series   Rangoon Recovered, which was derived from Rangoon Rough (2a), and found to have altered in its make-up from protein II to protein I and to have become smooth, stable, and agglutinable with Rangoon Smooth antiserum, the changes have come full circle, and the metabolism approximates that of the group I vibrios   Aerobic glycolysis has been completely recovered

In the case of the Rangoon strains, variations in metabolism accompany variations in structure, group, and serological and biochemical properties

TABLE III

The metabolism of original and variant strains of vibrios †

Strain	Group	Respiration c mm O_2	Aerobic glycolysis c mm CO_2	Anaerobic glycolysis c mm CO_2
Rangoon Smooth	I	1 34	0 65	2 13
Rangoon Rough (1)	I	0 83	0 47	1 81
Rangoon Rough (2)	V	0 30	0 0	0 0
Rangoon Rough (2a)	*	0 68	0 0	1 95
Rangoon Recovered	VI	0 97	0 83	1 99

* Glucose containing carbohydrate, protein not determined because of the instability of this strain

† 'Agglutinable' in this table indicates agglutination with antiserum prepared against living Rangoon Smooth vibrios, homologous titre, 1   25,600

TABLE III—*concld*

Strain	Group	Respiration c mm O_2	Aerobic glycolysis c mm CO_2	Anaerobic glycolysis c mm CO_2
El Tor I	IV	1 31	0 0	1 90
El Tor I-R	V	0 54	0 17	0 27
El Tor II	IV	1 01	0 0	2 04
El Tor II R	V	0 50	0 0	0 33
W880 (non-agg)	III	0 48	0 30	2 12
W880 (agg)	II and VI	0 96	0 84	2 10
10405	III	0 52	0 19	2 14
10405 T	II and VI	1 00	0 70	2 40
10405 (agg)	VI*	1 20	0 71	2 06
10405 D	V	0 58	0 14	0 26

* Predominately group VI, but contains traces of the galactose- and arabinose containing carbohydrates

Field strains —				
1331 (agg)	II	0 72	0 58	2 11
1331 (non agg)	III	0 69	0 30	2 00
6380 (non agg)	V	1 30	0 40	2 00
6380 (agg)	VI	0 98	0 82	2 10
China strains —				
Inaba	I	1 26	0 84	2 21
China 5	VI	0 80	[0 50	2 04
China 14	VI	0 97	0 83	1 78
China 21	III and IV	1 20	0 60	1 96
China 22	III and V	1 00	0 70	2 00
China 23	III and V	1 15	0 70	2 20

El Tor variants —These two variants from two El Tor strains (El Tor I-R and II-R in Table III) were ' medusa-head ' strains derived from the smooth parent strains by culturing them in 10 per cent peptone water   As in the case of Rangoon

Rough (2), the development of the ' medusa-head ' type of colony was accompanied by the appearance in the strain of the glucose-containing carbohydrate, and thus these strains had changed their position from group IV to group V The following changes were observed in metabolism oxygen consumption had fallen to about half its previous value, aerobic glycolysis which was negative in the case of both the original strains had increased slightly in one case and remained negative in the other, and finally anaerobic glycolysis had been reduced to about 15 per cent of its value in the smooth parent strains These variants accordingly are similar in metabolism to other strains of the same type, and differ noticeably from their parents

W880 variant —W880 (Linton, Shrivastava and Mitra, 1934) is a group III vibrio isolated from the river in Calcutta By means of repeated passages through mice, Taylor and Ahuja (1935) derived from it an agglutinable form which was found in this laboratory to be of mixed groups II and VI Metabolically, the agglutinating derivative has twice the respiratory power of the original, and nearly three times its power of aerobic glycolysis It thus has come to resemble metabolically the strains of groups II and VI whose chemical constituents it shares It is unchanged in anaerobic glycolysis

Field strain 10405 *and its variants* —The original of this group of vibrios was strain 10405, which was isolated from a carrier in the field It possesses the same metabolic values as the group III strains in general 10405 T and 10405 Agg were smooth variants agglutinable with cholera antiserum, which the original strain was not the first derived by Taylor and Ahuja (*loc cit*) by repeated mouse passages, and the second in this laboratory by repeated passages through 0 5 per cent glucose broth As the table shows, the application of both methods not only brought about agglutinability, but changes in metabolism as well In 10405 T, a strain of mixed group, the respiration doubled, and it was agglutinable at 1 800 with Rangoon Smooth antiserum In 10405 Agg , a strain of group VI, it more than doubled and this strain was agglutinable at 1 12,800 with the same antiserum A further change is found in the aerobic use of glucose which was tripled after the variation toward smoothness, and approximated that found in groups II and VI Anaerobic glycolysis remained unchanged

The fourth strain in the group, 10405 D, was a ' medusa-head ' strain derived in this laboratory from the parent 10405 in order to see what changes would accompany variation toward the rough side in contrast to those found in 10405 Agg where the variation was toward smoothness As in the case of the other ' medusa-head ' strains, it was found to fall into group V It does not differ from its group III parent in respiration or anaerobic glycolysis, both of which are low in comparison to the group I strains and to 10405 Agg On the other hand, its anaerobic glycolysis is only about 10 per cent of that of the parent strain In all these characteristics it resembles the ' medusa-head ' group

Field strains —The status of these strains as true variants cannot in the nature of the case be considered to be as well established as in strains which have varied while under observation in the laboratory The two sets of strains, 1331 and 6380, were derived on different dates from the same individuals, and were found agglutinable and of one chemical group at one isolation, and inagglutinable and of another chemical group at the second isolation Accordingly they cannot be

considered as proven derivatives one of the other Strain 1331 of 18th December, 1934, was an agglutinable organism derived from a case of cholera, belonging to group II and with the metabolic activities found in other members of this group The non-agglutinating vibrio, derived a month later from the same case when convalescent, was a group III organism which showed almost no change in its respiration or anaerobic glycolysis, and a loss of about one-half its glycolytic activity under aerobic conditions

In strain 6380 the reverse occurred The organism first isolated was non-agglutinating, and group V chemically The agglutinating strain isolated two weeks later was group VI chemically, and was double the first strain in respect to aerobic glycolysis

In these two sets of variants, accordingly, changes in chemical structure and agglutinability were accompanied by changes in metabolism

The China strains —Nothing very marked is apparent from the study of the metabolism of these strains They belong to groups I and VI, which are very alike in metabolism, and to the mixed group in which metabolism also appears marked in every case

DISCUSSION

The work reported in this paper has shown that metabolism is most active in the group of vibrios isolated from cases of cholera and belonging to group I In the members of group II the metabolic activity is less than in the first group, and in group III is even less although these two groups overlap in respect to respiration The El Tor group IV is sharply marked off from the others by the fact that, while its respiration is equal to that of group I its aerobic glycolysis is negative This interesting result is also found to occur in three vibrios of the El Tor chemical structure which had been isolated in India It appears that the study of metabolism may prove of value in differentiating this group of strains, in addition to the chemical analysis They cannot be differentiated serologically by any methods yet used, or by the use of hæmolytic tests, since, for example, strain 676 is non-hæmolytic, although it coincides with the other, hæmolytic, members of the group in chemical structure and metabolism Because of this finding we cannot agree with the opinion of Gardner and Venkatraman (1935) that the hæmolytic test is a suitable method for differentiating the El Tor vibrios from the cholera vibrios

Group V is made up of organisms of diverse origin Respiration is as high as in group I but the aerobic glycolysis has only about one-third the value This disparity is not present in group VI which is made up entirely of vibrios agglutinable with cholera antiserum, and which probably cannot be differentiated from the vibrios of group I by present serological methods

In the group of organisms with mixed carbohydrate components, groups II, III, IV, V and VI are represented and it is obviously impossible to do more than present the data, as the relative proportions of each kind of organism are unknown Respiration is high in all, and aerobic glycolysis is more variable than in the first six groups The same may be said for the unclassified carrier strains, especially in respect to aerobic glycolysis

In the unclassified group, the three water vibrios have the same metabolic powers as the similar vibrios in group III, and can be placed provisionally in this

group   It is also probable that vibrio 454 can, as we have shown above, be regarded as a group I vibrio on the basis of its origin in a case of cholera, its galactose-containing carbohydrate and its rate of aerobic glycolysis, which appears to rule out its belonging to group IV, in which this power is not present

The ' medusa-head ' organisms, which have been grouped separately, show a lowering of metabolism in every respect   Their average respiration is 0 45 c mm and is the lowest we have encountered although it is approached by the group III vibrios with 0 59 c mm   Aerobic glycolysis is negative in three of the strains, and of lower values than we have found elsewhere in the other two strains, excluding of course the organisms of group IV, where it is absent   The organisms of this type are the only ones in our series in which there is a change in anaerobic glycolysis Their average is about 10 per cent of that found in the other groups

In general we have found a distinct correlation between metabolism, chemical structure, and source of the strains   The most active vibrios metabolically are those from cholera, i e , group I, followed by groups II and VI also largely, but not exclusively, from human sources   Less completely active are groups III and V, and group IV is set off from the others by the absence of aerobic glycolysis in it The least active strains are those which show the widest divergence from the group I type, namely the ' medusa-head ' organisms   These strains, which make but slight demands on their environment, would appear to be the most adapted of any vibrios to survive for long periods under unfavourable conditions and some evidence has been found in this laboratory to show that they are in fact more resistant to sunlight than smooth vibrios are (Linton, Mitra and Seal, 1936)   On the other hand all the ' medusa-head ' strains which we have isolated are of laboratory origin, and so far as we know none has been found under natural conditions   As a result it is impossible to suggest what function, if any, they have in cholera epidemiology

In the strains we have studied, changes from agglutinability to inagglu-tinability have always accompanied a lowered metabolic activity   In the reverse case, the metabolism is increased   When a series like the Rangoon or the 10405 strains is studied, the same process is apparent—metabolism falls with increasing roughness and rises with reversion toward smoothness and agglutinability with cholera antiserum   This result strengthens the evidence which indicates that the vibrios of different chemical groups have different rates of metabolism and the work in general confirms the validity of the vibrio groupings based on chemical structure

Summary

A study of metabolism in a series of sixty-seven vibrios has brought out the following points —

1   Metabolism is most active in the group I vibrios, somewhat less active in groups II, V and VI, and least active in group III and the ' medusa-head ' group

2   The El Tor group IV, which also contains vibrios of the same chemical structure isolated in India, is sharply marked off by the absence in it of aerobic glycolysis

3   As vibrios change in chemical structure and in classification, their metabolism likewise changes to conform to that of their new grouping

4   Rough strains have lower metabolism than smooth strains and inagglutinable strains less than agglutinable strains

5   There is a good correlation in the vibrios between source, chemical structure, and metabolism

6   The bearing of these results on the validity of the chemical groupings of the vibrios is pointed out

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FURTHER NOTES ON THE CHOLERA AND CHOLERA-LIKE VIBRIOS

BY

RICHARD W LINTON, PH D,

B N MITRA, M SC,

AND

S C SEAL, M B

(Cholera Inquiry, Indian Research Fund Association, All-India Institute of Hygiene and Public Health, Calcutta)

THIS paper continues the series of notes on the structure and reactions of the cholera vibrio and related organisms (Linton, Shrivastava and Mitra, 1934)

A SHORTER METHOD FOR DETERMINING THE CHARACTERISTIC SUGAR OF THE SPECIFIC CARBOHYDRATE OF VIBRIOS

The following typical experiment, done on water vibrio W880, illustrates the method which has been developed

The 48-hour growth of 25 bottles or Roux flasks was washed off in 0 5 per cent phenol solution, and centrifuged until free from agar   The organisms were taken up in 50 c c of distilled water and 3 c c of concentrated H_2SO_4 added giving a concentration of 6 per cent of acid   This amount was higher than that used in the hydrolysis of purified carbohydrate, where 3 per cent is sufficient   The organisms were hydrolysed under reflux in the boiling water-bath for 1 5 hours The reducing power of the whole hydrolysate was equivalent to 75 0 mg of glucose

The solid portion of the hydrolysate was removed in the centrifuge, and the liquid portion neutralized to Congo-red paper with sodium acetate, boiled with animal charcoal, and filtered   The water-clear filtrate was concentrated on the water-bath to a small volume, and an equal volume of alcohol added to precipitate out the inorganic salts   After a further concentration of the salt-free liquid to remove the alcohol, the reducing power was again determined and found to be equivalent to 14 0 mg of glucose

A phenylosazone was formed from the filtrate, and its melting point found to be 159°C   Known arabinose forms an osazone melting at 160°C , and arabinose has previously been found in this organism as the characteristic sugar

The following results have been obtained by this method, and in Table I they are compared with previous results where the alkali extraction method was used —

TABLE I

Strain	Number of flasks	Reducing substance, mg	M P of osazone, °C	Characteristic sugar	Characteristic sugar in alkali extraction method
W880	25	75 0	159	Arabinose	Arabinose[1]
Kohat Original	50	300 0	206*	Glucose	Glucose[2]
676	20	43 0	191	Galactose	Galactose[2]
Rangoon Recovered	25		211	Glucose	Glucose[3]
603	60	360 0	207	Glucose	Glucose[2]
El Tor II R	34	35 0	204	Glucose	Glucose[2]
1617	20	56 0	186†	Galactose	Galactose
2027	20	48 0	158‡	Arabinose	Arabinose[4]
3075	20	76 0	160‡	Arabinose	Arabinose[5]
M 1	25	80 0	208	Glucose	Glucose[2]
M 2	25	124 0	206	Glucose	Glucose[2]
10405 (agg)	20	60 0	206*	Glucose	Glucose[2]

*   Mixed with known glucosozone, m p 208°C
†   Mixed with known galactosozone, m p 186°C
‡   Mixed with known arabinosozone, m p 158°C

The amounts of reducing substance given in the table are those obtained from the whole hydrolysate before it had been treated with charcoal   The chief disadvantage of the method is that the suspension of whole bacteria in acid becomes very dark during hydrolysis, and while the yield of reducing substance is originally

[1]   Linton, Shrivastava and Mitra,   *Ind Jour Med Res* , 1934, **22,** p 309
[2]   Linton, Mitra and Seal,   *ibid* ,   1936, **23,** p 589
[3]   Linton, Shrivastava and Mitra,   *ibid* ,   1935, **22,** p 653
[4]   Linton, Mitra and Shrivastava,   *ibid* ,   1934, **21,** p 749
[5]   Linton and Shrivastava,   *ibid* ,   1933, **21,** p 379

much larger than with the alkaline extraction method, most of this is lost ' in the necessary decolorizing with charcoal   The method is accordingly not satisfactory for obtaining amounts of carbohydrate larger than those needed for the formation of phenylosazone, i e , from 10 mg to 20 mg   The chief advantages of the method are that the amount of bacteria is much smaller than that required by the usual method, and that the lengthy process of separation of carbohydrate by alkali treatment and subsequent precipitation with large amounts of alcohol is eliminated

By the use of the direct method we can determine the nature of the carbohydrate quickly and directly from the organisms themselves, and we have shown in the table that the characteristic sugar of the polysaccharide of the organisms studied is the same as that obtained by the hydrolysis of the separated and purified carbohydrate

REACTIONS OF ISOLATED VIBRIO PROTEINS IN ALKALI

In two recent papers (Linton and Mitra, 1934 , Linton Mitra and Seal, 1935) we reported on the ' A ' and ' B ' fractions extractable from vibrio proteins in dilute acid solutions   We have subsequently extracted the proteins in dilute alkaline solutions and have followed the changes which occur under aerobic and anaerobic conditions

METHOD

The 48-hour growth from 80 bottles was washed free from agar in the usual way, taken up in 200 c c of distilled water which was made alkaline to pH 7 5 with caustic soda, and placed at 37°C with toluene as a preservative   Forty-eight hours later the suspension, which had a strong fæcal odour was centrifuged and the fluid portion brought to the acid side by the addition of dilute HCl and treated with 1 5 volumes of alcohol   The precipitate was taken off, and examined for specific rotation and nitrogen content   The fluid portion, designated the supernatant, was tested for any free amino acids   A portion of the solid residue was taken for Hausmann's analysis and the remainder was again added to the alkaline solution and incubated   Samples were similarly taken at intervals as shown in the tables

In the anaerobic study, the method was similar, except that the solutions were kept in an atmosphere of nitrogen

The two vibrios worked with were 1617 from a case of cholera, and W880 from water

RESULTS

*Supernatants —*The supernatant fluids of the two organisms in aerobic solution, after removal of the coagulable protein by acid, gave at each time interval positive tests for indol, glycine, leucine, tryptophane, arginine and urea   Histidine, cystine and tyrosine were also tested for, but could not be detected

In the supernatants of the two organisms under anaerobic conditions none of these constituents could be found even after 27 days at 37°C

Acid precipitates —The results of the analyses are given in Table II

TABLE II

Analyses of precipitates of vibrio suspensions under aerobic and anaerobic conditions

Strain	Time interval	N content, per cent	Weight, g	Specific rotation, °C *
Aerobic, 1617	48 hours	8 4	0 32	−23 0
	96 ,,	8 6	0 35	−20 0
	6 days	7 2	0 35	−16 6
	16 ,,	9 9	0 35	0 0
Aerobic, W880	48 hours	8 2	0 37	−25 0
	95 ,,	7 4	0 39	−19 3
	6 days	8 8	0 23	−15 0
	16 ,,	9 4	0 33	0 0
Anaerobic, 1617	18 hours	12 9	0 10	−51 0
	66 ,,	12 9	0 13	−52 0
	8 days	13 1	0 09	−47 0
	27 ,,	12 9	0 04	−44 9
Anaerobic, W880	18 hours	13 0	0 00	−53 0
	66 ,,	12 8	0 16	−50 0
	8 days	12 9	0 07	−48 0
	27	13 1		−52 0

* After 2 hours' incubation at 37°C in N/2 alkali

As Table II shows, disintegration was much more rapid in both organisms under aerobic than under anaerobic conditions Under the influence of air the percentage of nitrogen was always lower, showing that more break-down of protein was occurring, the amounts of protein which were going into solution were also

greater at each time interval, and the specific rotation shows again that disintegration had occurred hardly if at all under anaerobiasis, although the time interval was 27 days as against 16 days in the aerobic experiments

Residues —The analyses of the residues are given in Table III

TABLE III

Strain	Time	Amide N, per cent	Humin N, per cent	Bases, per cent	Phosphotungstic filtrate, per cent	Total N, per cent	Nitrogen, per cent
Aerobic conditions							
1617	48 hours		4 2	22 5	67 2	..	13 2
	96 ,,	6 1	3 8	20 2	68 4	98 5	12 2
	6 days	5 8	3 5	19 1	70 5	98 9	11 8
	16 ,,	5 2	3 3	18 7	71 0	98 2	10 8
W880	48 hours	6 8	3 9	23 3	66 3	100 3	12 9
	96 ,,	6 5	3 6	21 9	67 9	99 9	12 7
	6 days	5 9	3 7	20 0	69 6	99 2	12 0
	16 ,,	4 8	3 0	19 1	70 2	97 1	11 0
Anaerobic conditions							
1617	18 hours	7 2	3 9	24 7	65 8	101 6	13 4
	66 ,,	6 6	3 6	23 0	66 0	99 3	13 0
	8 days	7 0	4 2	22 8	65 9	99 9	13 7
	27 ,,	6 8	3 9	21 1	66 7	98 7	14 0
W880	18 hours	6 5	4 1	24 1	64 6	99 3	13 6
	66 ,,	6 8	3 8	23 0	65 3	98 9	14 2
	8 days	6 6	4 4	22 8	66 2	100 1	13 5
	27 ,,	7 0	3 8	21 2	67 2	99 4	13 8

The figures given in Table III present the converse picture of Table II The figure for amide nitrogen falls definitely under aerobic conditions, indicating that the amide linkages are being destroyed The bases are similarly attacked The total nitrogen is definitely less as the experiment proceeds Similar changes cannot be found in the figures for the proteins under anaerobic conditions

In these experiments it was not found possible to separate a definite fraction from the proteins by alkali   The proteins from either cholera or water vibrios disintegrate under the influence of alkali (pH 7 5), and the process is much more rapid under aerobic than under anaerobic conditions   As far as these experiments go they indicate that protein disintegration under anaerobic, alkaline conditions, similar to those found in the intestine, is extremely slow

THE SURVIVAL OF ROUGH, 'MEDUSA-HEAD' AND SMOOTH VIBRIOS IN RIVER WATER

Water obtained from the Hooghly at Calcutta was centrifuged, put through a Seitz filter with an 'E-K' disc, and finally through a Berkefeld filter   The water was then put into small flasks in 100 c c amounts, made neutral to litmus and inoculated with 200 million vibrios grown on agar slopes and washed off with sterile river water   In this way the addition of peptone was avoided   Sub-cultures were made on agar, daily at first and later on alternate days

The five Rangoon strains were used, as follows —

> Rangoon Smooth,
> Rangoon Rough (1),
> Rangoon Rough (2),
> Rangoon Rough (2a),
> Rangoon Recovered

We have reported extensive work on these strains, their characteristics and chemical structure (Linton, Shrivastava and Mitra, 1935)

After exposure to sunlight for one hour, only Rangoon Rough (2) and Rangoon Rough (2a) were found to have survived   Twenty-one days later, during which time these two strains were exposed daily for one hour, the 'medusa-head' Rangoon Rough (2) was still actively growing, and the intermediate smoother Rangoon Rough (2a) was present only scantily   Beginning with the 26th day, the cultures were exposed for two hours daily and thirty-one days later the growth of both had disappeared

This experiment demonstrates clearly the much higher resistance to sunlight of the rough strain of the 'medusa-head' type than of the smooth strains or the slightly rough strain   Rangoon Rough (2a) has a considerable 'medusa-head' element in it, and this increased somewhat as the experiment went on

In a second experiment, five times as many vibrios were inoculated (1,000 million) into 100 c c of sterile river water   The cultures were then exposed as before, and it was found that all survived, and that the smooth strains tended to become rough   As in the usual disinfection experiments, it is probable that the sunlight destroys the bacteria gradually, and that a small number of them will continue to survive, although slowly diminishing in number, after the majority has been killed   In view of our other work, it appears possible that the rough forms which 'appeared' in the smooth cultures exposed to sunlight were always present, and that they did not suffer from the exposure as the accompanying smooth forms did   As the latter are destroyed, a gradual change appears to be taking place   That such a situation can come about only when 1,000 million

bacteria are present initially, but not when 100 or 200 million are present, is understandable from a consideration of the mass law which appears to govern the death of bacteria in disinfection experiments

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EXPERIMENTAL OBSERVATIONS ON CHOLERA VACCINE

BY

LIEUT -COLONEL J TAYLOR, D S O , M D , D P H , I M S

CAPTAIN M L AHUJA, M D , CH B , I M S ,

AND

JEMADAR GURKIRPAL SINGH, D T M , I M D
(Indian Research Fund Association)

A KEEPING PROPERTIES OF CHOLERA VACCINE

IT has usually been accepted that cholera vaccine should not be used more than six months after the date of manufacture Maitra and Ahuja (1932) found that even when stored at a temperature of 37°C the vaccine retained its agglutinogenic power to a very considerable extent for a period of at least one year and that the results of agglutination tests with the stored vaccine compare favourably with those obtained with freshly manufactured vaccine

We have carried out a series of protection tests in guinea-pigs with vaccine freshly prepared or stored for various periods in the plains of India including at least one hot weather period, inoculating the animals with three doses of our stock carbolized cholera vaccine at two days' interval and subsequently giving them an intraperitoneal infection with a smooth agglutinable heterogeneous strain of *V choleræ* The results of four successive tests of this nature are shown in Table I —

TABLE I

Test	Age and type of vaccine	Number of guinea pigs vaccinated	Number of guinea-pigs surviving	Percentage survival
1	Six months old	5	5	100
	One month old	5	5	100
	Controls (non vaccinated)	5	0	0

T~ABLE~ I—*concld*

Test	Age and type of vaccine	Number of guinea pigs vaccinated	Number of guinea pigs surviving	Percentage survival
2	Two weeks old, carbolized	10	10	100
	One year old, carbolized	10	10	100
	Fresh, boiled for two hours	10	9	90
	Controls (non vaccinated)	10	0	0
3	Two years	10	9	90
	One year	10	9	90
	Nine months	10	9	90
	Six months	10	10	100
	Controls (non vaccinated)	10	0	0
4	Twenty-four hours old, carbolized	5	5	100
	One month old, carbolized	5	5	100
	Nine months old, carbolized	5	5	100
	Controls	5	0	0

The results suggest that vaccines stored up to two years in the plains of India and exposed to hot weather temperature of 110°F or over give protection practically equal to freshly made vaccines The interesting point has been observed, in the case of one of these tests, that vaccine boiled for two hours gives a protection little inferior to that obtained by means of freshly prepared vaccine killed by carbolic acid and not heated

Although it is not possible to draw any absolute deduction from the results of tests carried out by the abnormal route of intraperitoneal infection in guinea-pigs which may be applied to human inoculation, the observations, along with those previously made on the basis of agglutination response, would suggest that the cholera vaccine will retain its immunizing value for a much longer period than the six months now laid down as its expiry period and this without any special precaution such as preservation in cold storage

B Rate of development of immunity after inoculation

In the sanitary regulations of many countries it is provided that persons inoculated against cholera may obtain relief from the imposition of certain quarantine measures In the recently framed International Sanitary Convention for Aerial Navigation it is laid down that ' persons producing proof that they have been vaccinated against cholera within less than six months and more than six days may only be subjected to surveillance' This is presumably based on the assumption that effective immunity is produced within six days after inoculation While it is generally recommended that anti-cholera inoculation should be carried out by means of two or three doses, in practice, especially when mass inoculation is being done, a single dose is very frequently employed Practical experience has shown that single dose inoculation may be effective and the Office International d'Hygiene Publique has accepted that a single dose of inoculation may fulfil the requirements provided that a sufficiently high dose be employed We have investigated the rate of the development of immunity in animals after inoculation of a single dose, employing guinea-pigs for this purpose and, after immunization, injecting them intraperitoneally with a heterologous smooth strain of *V cholerœ* A series of fifteen guinea-pigs were each given 0 5 c c of the anti-cholera vaccine in routine manufacture at this Institute and the infecting dose was given at different intervals after inoculation to successive batches of five each with the following results —

Number of days after inoculation	Number of guinea pigs inoculated	Number of guinea pigs survived	Percentage survival	Number of controls inoculated	Number of controls survived
5	5	Nil	Nil	2	Nil
7	5	2	40	2	Nil
10	5	5	100	2	Nil

In this case it is seen that no effective immunity was produced by the fifth day, a partial immunity by the seventh day and complete protection by the tenth day

An interesting confirmation of the period taken for immunity to develop was obtained owing to a laboratory accident In connection with certain studies on

variation in vibrios, the Inaba strain, which may be taken as a classical example of the Japanese ' original ' type, and which corresponds serologically to the majority of agglutinable strains isolated from cases of cholera throughout the world, had been given a series of six intraperitoneal passages in white mice   As a result of this treatment it had become inagglutinable in regard to both its ' H ' and ' O ' fractions   While carrying out a further sub-passage of the inagglutinable variant the junior author (G S) accidently injected a portion of a heavy living suspension of the inagglutinable variant into his own wrist   On the same evening there was swelling and tenderness of the part, accompanied by a rise in temperature and malaise   The temperature fell on the following morning but the swelling increased and extended, subsequently disappearing in the course of three or four days   The serum of the victim of the accident was examined for its agglutination of the original Inaba agglutinable strain and also of the inagglutinable variant which had been injected   The following table shows the results of the tests at different intervals —

TABLE II.

Number of days after inoculation	TEST STRAINS			
	Inaba ' original '		Inaba ' variant '	
	' H '	' O '	' H '	' O '
2				
4			25	25
6	125		2,500	2,500
8	125		5,000	5,000
11	125		2,500	2,500
13	125		2,500	2,500
20	125		1,000	1,000

Although reaction in this case was not to an agglutinable cholera strain but to a variant, we may presume that a corresponding reaction would have occurred if the original strain had been injected   If the development of agglutinins can be taken as an indication of development of immunity a considerable degree of protection can be assumed to have been obtained by the sixth day and maximum degree of immunity by the eighth day

For practical purposes the adoption of a six-day period for quarantine regulations is probably suitable although it appears that a higher immunity would be obtained in eight to ten days

It is interesting to note that the very earliest use of a cholera vaccine by Ferran (1885) was based on the employment of living cultures   The agglutination response in human beings to an inoculation with a single dose of 1 c c of cholera vaccine containing 8,000 million vibrios is always at a much lower level than that which we have noted in this case of inoculation with a living suspension but, of course, the dose of living organism given was not measured

We have found single dose inoculation of carbolized vaccine to produce titres up to 1 in 250 only as a maximum in human subjects and as a rule the highest level was not reached before the tenth day

C   THE RELATIVE VALUE OF STRAINS FOR CHOLERA VACCINE

Employing the same technique for protection tests in guinea-pigs as was used when determining the keeping properties of the vaccine the animals were immunized with vaccines prepared from a variety of strains of different sources of origin These included strains showing both ' H ' and ' O ' agglutination, ' H ' agglutination only, and others which were inagglutinable with a cholera high-titre serum prepared against a strain corresponding to the Japanese ' original ' type   The strains were isolated from cholera cases, from cholera carriers and from water, and 'phage-derived variants were also used   In all tests the same heterologous strain of V *cholerœ* was used for the intraperitoneal infecting dose   The results of six groups of protection tests are shown in Table III —

TABLE III

Test number	Strain number	Origin	Linton's chemical group	Agglutination		Number of guinea pigs inoculated	Number of guinea-pigs surviving	Percentage survival
				' H '	' O '			
I	1617	Cholera case	I	+	+	4	4	100
	454	,, ,,	I	+	+	5	5	100
	454 P	'Phage variant	I	—	—	5	2	40
	3075	Water vibrio	III	+	—	4	2	50
	Controls					5	0	0
II	454	Cholera case	I	+	+	5	4	80
	454 P	'Phage variant	I	—	—	4	2	50
	3075	Water	III	+	—	5	2	40
	Controls					5	0	0

TABLE III—*concld*

Test number	Strain number	Origin	Linton's chemical group	Agglutination		Number of guinea-pigs inoculated	Number of guinea pigs surviving	Percentage survival
				'H'	'O'			
III	1617	Cholera case	I	+	+	5	5	100
	454	,, ,,	I	+	+	10	10	100
	454 P	'Phage variant of 454	I	—	—	10	4	40
	Controls					10	1	10
IV	Rangoon Smooth	Cholera case	I	+	+	4	4	100
	2027	,, ,,	II	+	+	5	5	100
	880	Water vibrio	III	—	—	5	2	40
	Controls					5	0	0
V	505	Cholera case	II	+	+	5	5	100
	1676	,, ,,	I	+	+	5	4	80
	1800	,, ,,		+	—	5	3	60
	Kohat	Water	V	—	—	5	0	0
	Controls					5	0	0
VI	603	Carrier vibrio	VI	+	+	5	3	60
	683	,, ,,		+	+	5	5	100
	676	,, ,,	IV	+	+	5	3	60
	17	,, ,,		+	+	5	3	60
	1312	,, ,,		+	+	5	4	80
	158	,, ,,	III	—	—	5	1	20
	508	,, ,,		—	—	5	5	..
	390	,, ,,		—	—	5	0	0
	467	, ,		—	—	5	1	20
	952	,, ,,		—	—	5	2	40
	950	,, ,,		—	—	5	1	20
	Controls	,, ,,		—	—	5	0	0

Throughout the series of tests it is shown that the highest degree of protection is given by vaccines prepared from strains which show both ' H ' and ' O ' agglutination with cholera high-titre serum   A much lower degree of protection was given by strains which showed ' H ' agglutination only   The survival rate in the case of animals inoculated with vaccines prepared from inagglutinable vibrio strains was of a low order   We have found that a survival rate against infection with *V cholerœ* as high as 40 per cent may be obtained after inoculation with T A B vaccine, so that any such rate of survival may be due to non-specific action

The majority of the agglutinable cholera strains used belong to Linton's chemical groups I and II and in most cases a 100 per cent survival was obtained with these strains

The infecting strain used belonged to chemical group I and vaccines prepared with both group I and group II vibrios protected equally against that strain   The vibrios of these two chemical groups form the great majority of strains isolated from cases of cholera in India and although a difference exists in regard to their carbohydrate fraction we have found them indistinguishable serologically by cross-absorption tests   The results of the present protection tests do not indicate any necessity to include strains of both group I and group II vibrios in anti-cholera vaccine as both appear to protect equally

Strain 454 P which is a 'phage-derived inagglutinable variant of the agglutinable cholera strain 454 is one which has undergone carbohydrate loss in the process of variation although such fraction of carbohydrate as remains is of similar type to that of the original form   This loss of carbohydrate can, in analogy with what is found in the case of the Salmonella group, be considered to account for its failure to agglutinate and would also explain its failure to protect [*]

Strains belonging to chemical group III have given little or no protection whether inagglutinable or showing ' H ' agglutination only

Two agglutinable strains of chemical groups IV and VI gave a slight degree of protection   The chemical structure of the agglutinable strain 683 was not determined

The protection given by vaccines made from agglutinable ' carrier ' strains, independent of their chemical structure, was of a much lower order than that obtained by vaccines of agglutinable cholera case strains

In previous communications (Taylor and Ahuja, 1935a and b) it has been shown that certain inagglutinable vibrio strains had become agglutinable after serial passages through animals   Three of these strains in their original inagglutinable form and in their subsequent agglutinable forms were used for the preparation of vaccines and comparative protection tests carried out with them   The results are shown in Table IV   The *V metchnikovi* strain in its original form as received by us from the Tropical Institute, Hamburg, was presumably not of cholera origin and gave practically no protection, but the passage strain which serologically was indistinguishable from *V cholerœ* gave 90 per cent survival in guinea-pigs   No 880 water strain gave no protection in its inagglutinable form but the

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[*] This strain has since undergone serological changes in our hands and in those of other workers

agglutinable passage variant gave 80 per cent   The inagglutinable carrier strain 10405 and its agglutinable passage variant gave respectively 30 per cent and 60 per cent survival

TABLE IV

Protective value of inagglutinable and agglutinable variants of vibrios from non-cholera sources

Strains	Agglutination		Linton's chemical group	Number of guinea pigs inoculated	Number of guinea pigs survived	Percentage survival
	'H'	'O'				
Metchnikovi 'original'	—	—	V	10	1	10
Metchnikovi 'passage'	+	+	V	10	9	90
Water 880 'original'	—	—	III	10	Nil	Nil
Water 880 'passage'	+	+	VI	10	8	80
Carrier 10405 'original'	—	—	III	10	3	30
Carrier 10405 'passage'	+	+	II and VI	10	6	60
Controls (non vaccinated)				10	Nil	Nil

The full protection given by vaccine made from cholera case strains was not obtained with these artificially derived agglutinable strains   The change from inagglutinability to agglutinability was, however, accompanied by a definite development of protective properties   In the case of the *V metchnikovi* strain this occurred without apparently any change in the nature of the chemical fractions although some increase in carbohydrate content had developed   A chemical change had occurred in the case of the passage variants of 880 and 10405 but none of the agglutinable variants showed the chemical composition associated with the group I and group II strains which had given the highest degree of protection in the previous series of tests

From these observations one can conclude that strains to be used for the preparation of anti-cholera vaccine should be those which show both 'H' and 'O' agglutination and are also of the chemical structure of which Linton (1935) has found to be characteristic of the majority of agglutinable strains isolated from clinical cases of cholera in India, i e , strains of chemical groups I and II   Our work has been carried out in relation to infection with strains of such type   The incidence of infection in India with vibrios of serological types which have been designated as Japanese 'variant' and Japanese 'intermediate' has not yet been determined although this point is at present under investigation   If infections with such strains are found to occur with any frequency it will be necessary to ascertain whether the strains which we have used for vaccine and which correspond to Japanese 'original' type will afford adequate protection against them

SUMMARY

1   Anti-cholera vaccine stored in India for periods up to two years, including exposure to hot weather temperatures in the Punjab, has been found to afford protection to experimental animals equal to that obtained with freshly prepared vaccine

2   As judged by the results of protective tests in animals and by the rate of development of agglutinins there appears to be evidence that immunity is not effectively developed after inoculation with anti-cholera vaccine before the fifth or sixth day   A higher degree of immunity is developed by the eighth to tenth day

3   The maximum degree of protection in animals against infection with strains of the prevailing serological type is obtained by the use of vaccine prepared from strains which show both ' H ' and ' O ' agglutination with a serum of the Japanese ' original ' type and which also show the chemical structure (Linton's chemical groups I and II) characteristic of the majority of agglutinable vibrio strains isolated from cases of cholera in India   Agglutinable strains from carriers and agglutinable variants produced from strains of origin other than cholera cases give a lower degree of protection

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A COMPARATIVE STUDY OF CERTAIN SELECTIVE MEDIA USED IN WATER ANALYSIS TOGETHER WITH A REVIEW OF THE LITERATURE ON THE SUBJECT

BY

T N S RAGHAVACHARI, B A ,

AND

P V SEETHARAMA IYER, M A

(*From the King Institute of Preventive Medicine, Guindy, Madras*)

CONTENTS

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* *Note* —Papers already published   Summaries are however, included in this paper for completeness of the comparisons

1 INTRODUCTORY

A SAFE water for human consumption is one which is free from harmful constituents, chief amongst which are the organisms known to cause disease The most rational procedure, therefore, to indicate the safety of a water would be to determine the presence or absence of specific pathogenic bacteria e g , those of typhoid, cholera, dysentery, etc , in the water Unfortunately, this task is not ordinarily possible in routine work and, even if it were possible, the chances of detecting the organisms are very remote It becomes necessary, therefore, to have recourse to some indirect index of the pollution present in the water, with the aid of which one may reasonably predict the probability of pathogenic organisms also being present Since the infections that are conveyed by water are primarily of intestinal origin, the presence of bacteria normally inhabiting the intestinal tract is taken as evidence of the potential danger of the water A number of different tests, chemical and bacteriological, have been in use for determining the presence in water of pollution of intestinal origin

A great deal of valuable information relating to the sanitary and hygienic quality of waters is obtained from chemical analysis, but it does not necessarily follow that a water which is quite satisfactory from the chemical point of view is or must necessarily be free from dangerous bacteria ' The delicacy of bacteriological methods is far greater than that of the chemical analysis ' (Thresh *et al* , 1933) The bacteriological examination is particularly delicate from the reason of its being very sensitive in detecting intestinal organisms when they are present and from the ease with which the tests may be done An organism to be considered an ideal index of fæcal pollution should satisfy the following requirements, viz , (*i*) it should be characteristically of fæcal origin, being derived from the intestines of men or animals, (*ii*) it should be relatively rare in nature outside the intestinal tract, (*iii*) it should be easy of rapid detection by simple methods, (*iv*) its incidence in water-supplies should bear some definite and constant relation to the bacteriologist's knowledge of the local conditions and to a sanitary survey carried out at the time of sample collection, (*v*) it should be distinctly more viable and more resistant in water and to treatment than the intestinal pathogenic bacteria (*B typhosus*, *V choleræ*, etc), but not excessively so (Levine, 1921*a* and *b*) An ideal index such as the one outlined above is not yet available, in the absence of which, the consensus of opinion amongst water bacteriologists all over the world has favoured the selection of the colon group for this purpose These organisms are not, in themselves, dangerous to health, but are significant as indices of the probable presence of pathogenic micro-organisms as well The members of this group are not, however, restricted to the human intestinal tract but are also found in other warm-blooded animals There are amongst this group, also, some organisms which are not of fæcal origin but are often present in soil, in grain, in milk and in other situations These would constitute a limitation to the unqualified acceptance of all members of the colon group of bacteria as being objectionable and dangerous when present in water None the less, in practice it is necessary for the analyst to interpret his results mainly on the basis of the presence of the colon group of bacilli He should, therefore, be thoroughly well acquainted with the characteristics and peculiarities of the members of this group particularly with reference to their distribution, viability and differential reactions

2 HISTORICAL

The bacteriological analysis of water and the significance of the presence of lactose fermenting coliform bacteria have been studied by numerous bacteriologists all over the world during the past 30 years A very large volume of literature has thus accumulated on the subject Much of the research work relates to the methods and media employed in routine water analysis Following the classical work of MacConkey in England, media containing bile salts have been in universal use in England In America, media containing bile in some form appear to have been largely used prior to 1912, when they were discarded in favour of the 'standard lactose broth' devoid of any inhibiting substance, as the bile was believed to be too inhibitive to the colon group of organisms

Within a few years, however, after this change was introduced, the standard lactose broth was found to be unsatisfactory as a preliminary enrichment medium , a very large number of spurious presumptive positive tests resulted from its use This involved a considerable expenditure of media, time and labour as all the presumptive positive tubes had to be confirmed on solid media

The spurious positives were found to be due mainly to the sporulating and non-sporulating aerobes and anaerobes which grew and fermented lactose, in the absence of an inhibitory agent in the culture medium In some cases, the false positive tests were due to symbiosis In their attempts to eliminate or minimize the above trouble, American bacteriologists have tried and advocated the use of various dyes and chemicals, alterations in the pH values of the preliminary enrichment media by buffering or otherwise, and the incubation of the primary cultures at 46°C The initial work on each of these lines has subsequently been either confirmed or refuted by workers in various parts of the world, e g , *Media containing chemicals or dyes* Phenol broth and phenol agar (Irons, 1901, Reynolds 1902, Muhiuddin Ali, 1932 , Dienert and Etrillard, 1930) Aesculin broth and agar (Harrison and Vanderleck, 1909 , Hale and Melia, 1911) Boric acid broth (Levine *et al* , 1934) Telluric acid medium (Chalmers, 1934) Liver broth (Jackson and Muer, 1911) Brilliant green bile (Jordan 1927) Basic fuchsin broth (Ritter, 1932) Crystal-violet (Salle, 1929) Gentian violet (Churchman, 1912, 1921, 1923) Methylene-blue and brom-cresol-purple broth (Dominick and Lauter, 1929 , Raghavachari and Seetharama Iyer, 1934) Congo red agar (Gaussen, 1928 , Fleury, 1932) Ferrocyanide citrate agar (Tonney and Noble, 1931) *Media with pH increased to 8 0 instead of 6 4* (Janzig and Montank, 1928) *The Eijkman fermentation test involving the incubation of cultures in glucose broth at 46°C* (Eijkman, 1904 , de Graaf, 1928 , Leitei, 1929 , Taylor and Goyle, 1931 , Brown and Skinner, 1930 , Ruchhoft *et al* , 1931 a, b and c , Kingsbury, 1932 , de Maghalhæs, 1932 , Williams *et al* 1933 , Perry and Hajna, 1933 , Webster and Raghavachari, 1934 , Webster, 1935)

It should be noted that not one of the newer methods enumerated has found universal acceptance for use in routine work

(a) *Bile salt and bile broths* —During recent years, considerable attention has been paid to the addition of bile to the media used in water analysis MacConkey (1900) first used bile salt (commercial sodium taurocholate) for the differentiation of *B coli* and *B typhosus* in fæces MacConkey and Hill (1901) used this medium with great success for the isolation of sewage bacteria Jackson (1907) reported

consistently satisfactory results with a bile medium in the examination of over 5,000 samples of water He found that the restraining action of bile salt was selective, favouring the growth and increase of *B coli,* retarding the growth of certain streptococci and actually killing off the majority of other species growing at 37°C Jackson and Muer *(loc cit)* further stated that all the known members of the typical *coli* group, gave positive gas tests with lactose bile, while no other known species gave such a result except *B welchii,* a pathogenic bacterium also of intestinal origin, which can be readily distinguished from *B coli* by microscopic examination after culturing in lactose bile broth Hale and Melia (1910) found that bile salts in lactose bile broth caused an appreciable degree of inhibition, in the development of the *B coli,* which increased with the attenuation of the organisms, that rejuvenation in suitable media, followed by transplanting to lactose bile broth, sometimes proved the presence of *B coli* not shown by the lactose bile broth in direct plantings, and that lactose bile broth gave more reliable presumptive tests for *B coli* than any other known medium, including Aesculin broth Other species of bacteria caused much less interference with gas formation by *B coli* in lactose bile broth than in other media Lactose bile broth was of particular value in making a distinction between recent and more remote pollution and gave more satisfactory evidence of the actual relative sanitary and hygienic qualities of water

Jackson and Muer *(loc cit)* showed that lactose bile broth was slightly inhibitive only to the attenuated forms of *B coli* in water, so that any positive tests with lactose bile broth indicated recent contamination

Jordan (1913) used fresh ox-bile and found that it inhibited at least from one-third to one-half of the viable cells of *B coli* and sometimes a much larger proportion Freshly isolated cultures were inhibited in at least the same degree as those under long cultivation or those subjected to a prolonged sojourn in water He found no evidence that *B coli* organisms which were unable to grow on bile media were any more attenuated or less vigorous biologically than their fellows and that, with some care in the technique, *B coli* could be isolated in a larger proportion of cases from standard lactose broth tubes than from lactose bile broth tubes He concluded that bile was strongly inhibitory to *B coli* and that its use always involved a suppression of a certain number of viable bacteria, which cannot be assumed to be insignificant in the interpretation of water analysis

Bunker (1916) showed that a medium composed of 1 per cent peptone, 1 per cent lactose and 2 per cent to 5 per cent of dried bile was more delicate in detecting intestinal organisms, and was, in the majority of cases more reliable than the standard lactose broth (devoid of any inhibitory agent)

Obst (1916) compared lactose bile broth with standard lactose broth and favoured the latter as it involved less expenditure of money, labour and time Pure fresh bile of uniform composition was difficult to get and stored bile showed progressive deterioration in strength and quality

Salter (1919) studied the effect of different concentrations of bile salt on the rate of growth of *B coli communis* and found that small quantities of bile salts, i e , under five-tenths per cent, stimulated the growth of the organism while greater amounts, such as 1 per cent, greatly inhibited growth He emphasized the need for investigating the effect of varying amounts of bile salt on other members of the colon group.

Ritter (1919) examined 1,899 samples of surface water using lactose bile broth and standard lactose broth and found that (1) when lactose bile broth and standard lactose broth were both fermented the presumptive test was reliable in 75 per cent of all cases considered, (2) if all three broth tubes in a series (as well as lactose bile broth) were positive, a greater percentage of tests was confirmed for *B coli*, than was the case when only one or two tubes were positive, (3) when the standard lactose broth alone was positive, in 70 per cent to 78 per cent of cases the water was proved *not* to contain *B coli*, (4) and samples positive in standard lactose broth in 24 hours contained *B coli* in 97 7 per cent of cases and therefore required no confirmation

Muer and Harris (1920) proved that lactose bile broth containing 5 per cent dried ox-gall was preferable to a 10 per cent medium, as in the former (*a*) the formation of gas was more rapid and greater in amount, (*b*) positive tests were obtained in higher dilutions, (*c*) attenuated forms of *B coli* were more readily demonstrated, (*d*) interference by other bacteria, especially the streptococci, was less evident, (*e*) a clearer medium, free from sediment, was available for use and (*f*) forcing out of cotton plugs and foaming did not occur in the process of sterilization of the medium

Schœnholz and Meyer (1921) demonstrated that the influence of bile salts upon the typhoid bacillus varied markedly at different pH concentrations At a pH of 7 0, even 1 per cent bile increased growth while at pH of 8 0, even 0 5 per cent was inhibitive

Levine (1921*a* and *b*) summed up the arguments for and against the use of bile for presumptive tests as under (1) lactose bile broth is more reliable for presumptive tests but a greater proportion of the colon group may be detected by preliminary enrichment in standard lactose broth, (2) if the proper concentration of bile salts could be determined, the lactose bile broth would probably be preferable to standard lactose broth It is very probable that if a standardized evaporated bile were available, a concentration of 1 per cent to 2 per cent in lactose peptone water would be superior to the standard lactose broth

Levine (1922) compared sodium taurocholate (Merck) with evaporated bile (Difco) as regards their effect on the rate of multiplication of *B coli* and *B cerogenes* He found an optimum concentration of 1 5 per cent of sodium taurocholate to accelerate the growth of *B coli* and *B cerogenes* Higher concentrations than 1 per cent of Difco dried bile were inhibitory He favoured the inclusion of 1 per cent to 2 per cent of dried bile for preliminary enrichment medium for the colon group of organisms He found that such a medium favoured the acceleration of the growth of *B coli* and *B cerogenes* and inhibited many of the anaerobic spore-forming lactose fermenters Spore-forming aerobic lactose fermenters did not grow in peptone lactose broth containing bile For comparable results a standard evaporated bile or pure bile salts were considered essential

Winslow and Dolloff (1922) tested five strains of colon organisms using sodium choleate and found that bile salt lactose broth was distinctly more favourable than the standard lactose broth without bile since at every pH value the former showed more gas in 48 hours than the latter at 96 hours and the amount of gas progressively increased on increasing the alkalinity of the medium up to pH 8 0

Houston (1920–1933) used MacConkey's bile salt lactose broth in his classical research work on water extending over several years

Cunningham and Raghavachari (1924, 1926) and Raghavachari (1926) found MacConkey's bile salt lactose broth to be a most suitable medium for the isolation and differentiation of lactose fermenting coliform bacteria from fæces, soil, milk and water

Dunham *et al* (1925), the referee appointed by the Committee No 1 on Standard Methods of Water Analysis, stated (*i*) that evaporated bile may inhibit or stimulate the growth of the *coli* group depending on concentration and reaction, (*ii*) different samples of bile varied in their inhibitory or stimulating effects, (*iii*) with all samples of bile tested, a 2 per cent concentration was found to stimulate growth, (*iv*) in an alkaline range of pH 7 3 to 7 8 five per cent bile stimulated growth, while at pH 6 1 considerable retardation resulted, (*v*) the optimum reaction for growth of *B coli* is a function of the composition of the medium, thus, in peptone lactose broth *without* bile, growth was best at pH 6 1, while in lactose bile broth the most favourable range of pH was between 7 3 and 7 8

Holwerda (1928 1930) reported excellent results with the use of MacConkey's bile salt lactose broth in his researches on coliform bacteria in fæces, soil and water His results were confirmed by de Roode (1928) in an independent investigation using the same medium

Cameron (1930) used a modified lactose bile broth on chlorinated water samples at Fostoria and found that it eliminated a little over 90 per cent of the gas formers as determined in standard lactose broth and did not inhibit or interfere with the presumptive test In fact he found the presumptive index was about 60 per cent higher in the lactose bile broth In the case of the standard lactose broth there were 405 ten-c c cultures containing gas formers, whereas in the lactose bile broth there were only 40 ten-c c cultures showing gas that had to be confirmed

Ey (1930) compared the modified lactose bile broth and the standard lactose broth by testing 50 selected well waters Twenty-four were negative in both and 26 showed *B coli* by one or both methods Of these 26, seventeen or 65 per cent showed gas in 48 hours by both methods and 14 proved to be true *B coli* The other three gave suspicious colonies Five or 19 per cent showed gas in standard lactose broth, only four of which confirmed Four or 15 per cent showed gas in lactose bile broth only, of which only one confirmed He would *not*, therefore, accept modified lactose bile broth as the final solution until more research work had been carried out, using both media

Ruchhoft *et al* (*loc cit*) suggested that a 2 per cent bile medium may be buffered and the pH adjusted to 7 6 to 7 8, and rendered less inhibitive and relatively less selective

Evans and Bahlman (1931), working with samples of raw settled water, showed that the percentage of modified lactose bile broth tubes showing gas was 29 1 as compared with 22 2 for the standard lactose broth, the percentage of confirmations being 21 1 and 19 0 respectively The percentage of presumptive positives that confirmed was 71 4 and 85 7 respectively

Gettrust and Hostettler (1930, 1931) carried out comparative tests at five different water purification plants, using standard lactose broth and a modified lactose bile broth (3 57 grammes of ox-gall per litre of standard lactose broth)

The data obtained indicated that the modified lactose bile broth inhibited most of the false presumptives met with in filtered and chlorinated waters Tests on raw waters showed more gas formers and more confirmations with modified lactose bile broth than with the other

Lochhead and Hewer (1931) compared standard lactose broth with lactose bile broth (Difco dehydrated products being used in both cases) on 482 samples of water Positive tests were in each case confirmed by transfers to eosin methylene-blue agar, two isolated colonies, considered to be fæcal, being examined by the usual further tests and differentiated into *coli* and *ærogenes* Of 2,410 tubes of each medium inoculated, 1,263 and 1,199 tubes respectively showed gas formation *Coli-ærogenes* organisms were recovered from 649 of the former and 782 of the latter They concluded that there was no advantage in using lactose bile broth, if the presence of the *coli-ærogenes* group was to be considered as the criterion of pollution but that the lactose bile broth was to be preferred if the fæcal type of *B coli* alone was to be regarded as the index of pollution

Bole (1931) reported on a series of comparative tests on well and spring-well waters, using standard lactose broth and a modified lactose bile broth (3 5 grammes of ox-gall alone being added to each litre of the standard lactose broth) The percentage of presumptive positive tubes confirmed for *coli-ærogenes* was 94 8 and 96 6 respectively The inhibiting effect of bile was confined to the *ærogenes* group *B coli* completions being increased Parallel use of the standard lactose broth and the lactose bile broth gave nearly 100 per cent completion when gas was present in both There was 6 per cent of the total completions by both methods which did not ferment the standard lactose broth and 12 per cent which did not ferment the modified lactose bile broth

Pawan (1931) used MacConkey's bile salt lactose broth with success in his research work on fæces and water

Hirst (1932) reported very good results using MacConkey's bile salt lactose broth on the Colombo water-supply

A careful analysis of the literature summarized above indicates that (1) the use of the standard lactose broth in routine water analysis has not been satisfactory, as it gives rise to a considerable number of false presumptive positive tests, (2) bile in some form or other has been found to inhibit the spurious gas formers, and (3) the concentration of the bile in the medium and the reaction to which the medium has to be adjusted are the most important factors controlling the success or failure of this medium The pH value of the lactose bile broth should be maintained always at 7 4 to 7 8, while that of the standard lactose broth should be at 6 4 to 7 0 The inhibition of attenuated forms of *B coli*, which is stated to be associated with the use of bile, has been the subject of adverse criticism by a few of the workers who consider that the presence of these forms in water is not less significant than that of the viable forms The bulk of informed opinion on this subject tends, however, to the view that the attenuated forms may be safely ignored in sanitary water analysis

Most of the observers cited above have used either fresh bile or dried ox-bile In these two forms, bile has not yielded consistently uniform results By using a standardized evaporated bile (Difco product) it was found that the true *B coli* of

fæcal origin were more readily brought out, which furnished adequate evidence of the presence of comparatively recent and potentially dangerous fæcal pollution in water

We have not been able to ascertain from the American literature cited above, the reason why the commercial sodium taurocholate (bile salt) advocated by MacConkey in 1900, and used ever since, with uniformly good results all over the British Empire has not found favour in American practice   At the request of Dominick and Lauter, a small supply of the commercial salt used in our laboratory was made to them in March 1934, but their results on its use have not yet reached us   We are inclined to believe that had this salt been used in a concentration of 1 5 per cent (for making the stock broth) in preference to fresh or dried ox-bile, and had this bile salt lactose broth been adjusted to a pH of 7 4 to 7 8, the reports from the bacteriologists of the New World would have been all in favour of the bile salt   They would have had little or no trouble from the spurious presumptive positives which have been seriously interfering with their tests

(*b*) *Brilliant green bile broth* —Without giving this medium a fair trial in their comparisons, the American bacteriologists have been trying the use of various dyes either alone or in combination, mostly with bile (fresh or dried)   Brilliant green has been tried by a large number of workers in combination with bile Their findings briefly reviewed are as under —

Winslow and Dolloff (*loc cit*) used brilliant green along with bile salt and found that the dye inhibited the organisms studied in concentrations between 1   100,000 and 1   1,000,000 in the broth medium (*B œrogenes* was, however, slightly more resistant than *B coli*), but that in the presence of the bile salt, the toxicity of the dye wholly disappeared and *B œrogenes* and *B pneumoniæ* grew even in a concentration of 1   500 of the dye   They tried two other triphenylmethane dyes, viz , rosolic acid and gentian violet, but found them too inhibitive both alone and in combination with bile salt

McCrady (1925) in recording the findings by himself and his collaborators in other laboratories, on especially selected samples of brilliant green and dried ox-gall, concluded that brilliant green bile broth eliminated practically all spore-forming lactose fermenters and seemed to be well adapted for use with certain types of waters   This medium was, therefore, considered to have a certain well-defined sphere of usefulness

Jordan (1925) used brilliant green bile broth as a *confirmatory* medium and proved that (1) it eliminated the large number of Endo plates on which no growth occurred, (2) it eliminated spore-forming organisms, (3) it yielded a 99 5 per cent completion of brilliant green bile-broth cultures, (4) it did not inhibit any organisms of the colon group when used for confirmation, (5) the concentrations of brilliant green 1   10,000 and bile 5 per cent did not seem to be such as to be absolutely specific for the colon group

Howard and Thompson (1925) showed that broth made with the addition of bile salt and brilliant green was inhibitory to the colon group

Stearn and Stearn (1925) and, later, Rakieten and Rettger (1927) found that the inhibitive effect of brilliant green on Gram-positive organisms diminished as the pH was raised above 7 0

Ruchhoft (1926) considered that brilliant green bile broth was too inhibitive to the *B coli* group to be used for direct preliminary enrichment

Schoenlin (1926) observed the greatest stimulation of *coli* organisms occurring in 2 per cent bile, lesser stimulation down to 1 per cent and up to 3 per cent With 2 per cent bile the maximum favourable density of brilliant green was 1 75,000 and the optimum pH was 6 9

Lauter (1926) compared standard lactose broth with brilliant green bile broth In 1923, he found 51 per cent of tubes showing gas in the former and 73 per cent in the latter confirmed on eosin-methylene-blue agar He had 740 presumptive positives in brilliant green bile broth in 2 days and 741 confirmed in standard lactose broth after a minimum of 3 days, a gain of one day with exactly the same figure for treated waters On the raw water, he had 87 presumptives in 2 days against 81 confirmed in standard lactose broth in 3 days—a greater number of positives evidently, but on the safe side The check was very good and in favour of brilliant green bile broth giving a larger number of positives, which meant a greater tendency towards safety in watching a water It was much simpler to make brilliant green bile broth tubes than eosin methylene-blue agar plates and the presence or absence of gas in any desired volume was easier to record than a 'colour reaction which had proved itself off shade so much of the time'

Hale (1926) advocated the use of brilliant green bile broth as a straight presumptive test without further confirmation He would attach no significance to the differentiation of the colon group into *B coli* and *B aerogenes*, as a criterion of faecal pollution He found that brilliant green bile broth gave a reliable picture of the sanitary quality of a water-supply from source to consumer, that the amount of gas was usually greater in brilliant green bile broth than in standard lactose broth tubes, and that attenuation was the deciding factor in delayed gas formation in brilliant green bile broth He used a brilliant green bile broth containing 8 per cent ox-gall and the proportionate concentrations of lactose, peptone and brilliant green. His final dilutions after the inoculations of water had a 4 per cent concentration of bile

Koser and Shinn (1927) tested various combinations of brilliant green with a view to ascertaining the capacity of spore-forming lactose fermenters to grow in brilliant green bile broth By running suitable controls (without brilliant green) with only peptone and ox-gall, they showed that the inhibitive action was due to bile rather than to the dye

Dunham (*loc cit*), the referee appointed by Committee No 1 on Standard Methods of Water Analysis, summarized the results of the investigations on the composition of the brilliant green bile broth as under —

(1) The amount of brilliant green giving satisfactory results in bile media was proportional to the concentration of bile , with 2 per cent evaporated bile, concentrations of the dye of over 1 50,000 proved distinctly inhibitory With 5 per cent bile a concentration of 1 20,000 of the dye gave very good growth

(2) Two per cent bile was slightly superior to 5 per cent bile both as the preliminary enrichment medium and as a sub-cultural medium

(3) When brilliant green bile broth was used for direct planting, the percentage of original fermentations checked well with the previous studies of the Committee but the 2 per cent bile showed a slightly higher degree of completion and no cultures had to be discarded because of the presence of spore-formers

(4) When brilliant green bile broth was used as the second step in a sub-cultural series the degree of completion in the better bile density (i e, 2 per cent) was 95 per cent

(5) In 3 cases out of 1,487 cultures, the presence of spores was noted and used as a basis for rejecting the completion   This frequency of 0 2 per cent was considered unimportant   Dunham, therefore, advised the adoption of brilliant green bile broth as a secondary or supplementary procedure in routine water analysis

Bole (*loc cit*) considered that a combination of bile and brilliant green in optimum concentration would perhaps be the best, if still an incomplete answer to the problem of selective media in water analysis

Gettrust and Hostettler (1931) came to a similar conclusion

Jordan (1932) considered that the use of standard lactose broth, by the profusion of its false positive reactions, cast a doubt on many water-supplies and that, therefore it lacked specificity and was of little or no value as an aid to the water-works operator   He, therefore, advocated the adoption of the parallel planting procedure, which has since been included in the recent edition of the standard methods   It is claimed, for this procedure, that the use of brilliant green bile broth gives a prompt indication of massive pollution and at the same time permits the standard lactose broth to rejuvenate organisms whose metabolic activity is at a comparatively low level   Time and specificity are thus believed to have been gained and the major function of an operating laboratory protecting the public is greatly facilitated by a reasonable blending of promptness and accuracy

Wells (1932), an advocate of Jordan's parallel planting technique, has elaborated an automatic transfer device consisting of a twin battery of fermentation tubes connected by capillary siphon which serves to inoculate secondary tubes of brilliant green bile broth just as gas begins to appear in the primary standard lactose broth tubes

Keatley (1933) found that brilliant green bile broth eliminated false presumptive positives which were of frequent occurrence with the standard lactose broth

Parr and Caldwell (1933) instituted a comparison by direct inoculations in brilliant green bile broth (2 per cent) and in standard lactose broth of 1,407 samples of water and found that brilliant green bile broth was much superior to the standard lactose broth as the former suppressed spurious positive presumptives   Their results indicated that the correlation of the recovery of the *colon aerogenes* group with production of gas in brilliant green bile broth was less perfect, in certain types of waters at least, than previously published work would indicate   Brilliant green bile broth was undoubtedly superior in most aspects to standard lactose broth

and when completed with lactose, as described in the tests proposed, would give highly satisfactory results, if the procedure stopped short of the 'completed tests' They urged finally the necessity for further tests on parallel planting and gave a warning against any attempt to discourage other selective media that might perhaps prove to be equally satisfactory

Butterfield (1933) compared two brilliant green bile broths, one containing 2 per cent bile with a brilliant green concentration of 1 75,000 and the other 5 per cent bile with 1 20,000 of the dye While the brilliant green bile broth presumptives confirmed in almost 100 per cent of the cases, they were not equally successful in detecting all bacteria of the *coli aerogenes* group in waters from different sources and so were considered unfit for use as standard media He concluded that a parallel planting procedure designed to combine the advantage of the productivity of the standard lactose broth with the restrictive qualities of the brilliant green bile broth was impracticable

Horwood and Heifetz (1934) considered standard lactose broth to be the best presumptive test medium, although nearly the same degree of sensitivity was obtained with the brilliant green bile broth They, therefore, strongly advocated the buffering of the standard lactose broth and making other changes to improve the same, before finally abandoning it as a presumptive medium

(c) *Media using other dyes and chemicals* —Another promising line of research along which a fairly large amount of work has been carried out during recent years relates to the use of various other dye-stuffs for inhibiting the spurious lactose fermenters

Churchman (*loc cit*) showed that gentian violet was very toxic to Gram-positive organisms but that it had little or no effect on Gram-negative organisms except in high concentrations The effect of the dye was proved to be bacteriostatic rather than bactericidal

Hall and Ellefson (1918) demonstrated that gentian violet, when added to the standard lactose broth, eliminated the false positives met with in water analysis, which were due to anaerobes They later (1919) recommended the use of this dye in routine water analysis, as the percentage of confirmations was also much higher in the dye broth than in the broth containing no dye

Similar conclusions were reached by Bernstein and Loewe (1919), Gay and Beckwith (1922), Norton and Davis (1923), Stearn (1923), Burke and Skinner (1925) and Weisner (1926)

Wagner and Monfort (1921) suggested the use of a medium (omitting beef extract) with 2 per cent peptone, 0 2 per cent lactose and 0 001 per cent gentian violet They recommended pasteurization instead of sterilization of the medium for ensuring good results, and they relied on the gentian violet for inhibiting the spore-formers that would normally resist pasteurization

Kessler and Swenarton (1927) advocated the use of a special medium for the detection of *B coli* in milk It was a modification of brilliant green bile broth using gentian violet instead of brilliant green and a lesser proportion of bile (1 per cent) One thousand and ten examinations for *B coli* revealed 1,000 positives for typical members of the colon group This method was, therefore,

considered by him to be suitable for water analysis, as it was superior to the standard lactose broth as evidenced by collaborative work in a water analysis laboratory

Poe and Witt (1930) estimated the comparative values of brilliant green and malachite green in 5 per cent bile broth and found that a dye concentration of 1 20,000 in the case of brilliant green and of 1 30,000 of malachite green was sufficient to inhibit most anaerobes in water

Gaussen (*loc cit*) advocated the use of toluidine blue in peptone medium for the presumptive test, as this medium gave a very reliable picture of the sanitary quality of a water

Fleury (*loc cit*) reported very good results with Congo red when used in a solid medium for direct plating

Ritter (1932) used basic fuchsin in a concentration of 1 1 250, and obtained 94 5 per cent confirmations from 33,532 presumptive tests as compared with 57 5 per cent in 45,030 tests with the standard lactose broth The selective action of fuchsin broth was found to be most marked in the case of chlorinated waters

Bronfenbrenner *et al* (1920) found that rosolic acid was a very dependable antiseptic for checking the growth and development of Gram-positive bacteria without interfering with the normal growth of the Gram-negative bacteria in water Even *B dysenteriæ*, which was inhibited by brilliant green and crystal violet, was resistant to rosolic acid

Salle (1929) showed that gentian violet was not a definite chemical compound but rather a mixture of various dyes belonging to the para-rosaniline series Crystal violet was one of the dyes entering into the mixture and had a definite chemical formula, viz, hexamethyl-para-rosaniline

Browning, Gilmour and Mackie (1913) and Browning *et al* (1914) demonstrated that crystal violet was one of the most active bactericidal substances known and that the amount required to inhibit the colon group was 1 500 Brilliant green was considered to be at least twenty times more toxic to *B coli* than gentian violet

Salle (1930) outlined a scheme of water analysis, and described a crystal violet broth which, he showed, was superior to the brilliant green bile broth and gave 100 per cent positive results for members of the *coli ærogenes* group

Stark and England (1933), however, discounted the value of Salle's crystal violet broth as they obtained poor results with its use on pure cultures

The use of brom-cresol-purple was suggested by Prescott in 1924 (Prescott and Winslow, 1931) Salle carried out further studies using this medium in 1929 Dominick and Lauter (*loc cit*) developed a methylene-blue brom-cresol-purple broth in which a sharp colour change from blue through green to yellow was claimed to be specific for *B coli* and so the presumptive test was absolute and needed no confirmation This medium has since found a number of adherents, prominent amongst whom are Leahy, Freeman and Katsamps (1931), McCants (1931), Stewart (1933) and Nolte and Kramer (1933) The latest issue of the American Standard Methods, 7th edition (A P H Association, 1933) has

included this medium and also Salle's crystal violet broth as non-standard media, worthy of being tried   Raghavachari and Seetharama Iyer (*loc cit*), however, showed that although the Dominick-Lauter broth yielded better results than the standard lactose broth, it was definitely inferior to the bile salt lactose broth of MacConkey both as a preliminary enrichment medium and as a sub-cultural medium

Howard (1932) compared the Dominick-Lauter broth with brilliant green bile broth and the standard lactose broth   He showed that the brilliant green bile broth was preferable to Dominick-Lauter broth as the latter depended for its value on the established suitability and purity of the dyes used in making the medium He stated that without a guarantee on this point the medium might be of but limited value

Norton (1933), in his very illuminating review of the literature and research work on the technique of water analysis, has emphasized the need for caution in using the newer media with dyes in their composition   Great care should be exercised in the final evaluation of water supplies when such dye-containing media are employed for inhibition purposes   They must be proved to yield results comparable with those obtained from a non-inhibiting medium on *the particular water-supply under consideration*

Levine *et al* (*loc cit*) suggested a new medium using boric acid with proteose peptone and lactose which is claimed to be specific for the true *B coli*, the *œrogenes* and *intermediates* being effectively suppressed by the boric acid   We have examined 24 samples of water so far, but our results do not substantiate the claim, as *B coli* and *B œrogenes* grow equally well in the new medium   We are continuing the comparison with a new modification since suggested by Levine and our results will form the subject-matter of a later note

Chalmers (*loc cit*) found that by using 0 C013 per cent telluric acid in a bile salt broth, a minimum inhibition of the true *B coli* and a maximum inhibition of the *B œrogenes* could be achieved   He showed that the telluric acid was, therefore, superior to brilliant green which allowed both *coli* and *œrogenes* to grow in its presence

(*d*) *Buffering of media and varying their pH* —Janzig and Montank (*loc cit*) obtained an elimination of 47 per cent of the false presumptive positives by using a lactose broth of decreased pH value   They claimed that this would result in a considerable saving in the time required to certify a water-supply   Thompson (1927) recommended the buffering of the standard lactose broth by the addition of 2 g of di-potassium hydrogen phosphate to each litre of broth for eliminating the spurious lactose fermenters

Ruchhoft *et al* (*loc cit*) claimed that buffering would prevent the production of a pH lethal for *coli-œrogenes* and thus make it possible to confirm more presumptives

Clark (1931) made parallel tests on 114 samples using lactose broths of two different pH values, viz , 6 8 and 8 1   Thirty-two samples gave negative presumptive positive tests in both broths   There was no appreciable reduction in the number of false presumptive tests and the lactose broth of pH 8 1 gave fewer isolations for *B coli*—11 per cent fewer—than the standard lactose broth of pH 6 8

(c) *Eijkman's test involving incubation of glucose broth cultures at 46°C* — Muhiuddin Ali (*loc cit*) advocated Vincent's method which depended on the growth of *coli* but of no other water bacteria at 41 5°C in 1 per cent peptone broth, containing 0 5 per cent sodium chloride and 0 083 per cent phenol He considered this medium to be very sensitive for vigorous young cultures, but older ones were more sensitive to phenol and the method would, therefore, not detect *coli* which had been in water for some days The positive tests with this medium would thus indicate recent pollution with great accuracy.

The Eijkman fermentation test, which involves the incubation of the preliminary enrichment cultures in glucose broth, at a temperature of 46°C , has been very extensively tried by numerous workers all over the world The results reported by them are extremely conflicting, from unqualified approval by some to unequivocal condemnation by others This test has not, therefore, met with acceptance for routine use, except in Germany, where, however, it is only one of three tests usually employed

The routine standard practice in America has been preliminary enrichment in standard lactose broth, and subsequent confirmation of only one of the *gas positive* tubes—usually the tube showing gas with the smallest volume of the inoculum—on eosin-methylene-blue agar for the presence of members of the *coli-aerogenes* group This is usually carried out by picking off one or two suspicious looking colonies from the plate, examining their staining reaction by Gram's method and, if they are Gram-negative, passing them again through the same standard lactose broth for gas production The parallel planting procedure outlined in the latest edition of the Standard Methods is not apparently meant to be adopted as a routine measure for all water examinations

The standard routine practice in England and the British Empire generally—including India, Burma, Ceylon, Hongkong, and all other tropical possessions in Africa and in the Far East—has been preliminary enrichment in MacConkey's bile salt lactose broth and reading off the results—acid and gas production—at the end of 48 hours' incubation at 37°C Confirmations are usually made only in the case of samples from sources under investigation or from sources whose antecedents are unknown

In France—and possibly also in all French possessions—preliminary enrichment in phenol broth and subsequent confirmation on phenol agar constitute the established routine procedure (Dienert and Etrillard *loc cit*)

In Germany lactose-peptone azolitmin solution, Eijkman's glucose-peptone solution, and Buhr's neutral-mannite bouillon are used for the *B coli* tests These media have received the official sanction of the Prussian Public Health Board (Schoetz 1932)

In Sweden, the direct plate count on Congo-red agar has been the favourite and officially recognized test in successful use for many years (Huss, 1926)

In Italy, Parietti's method of determining the *coli* index is considered the most sensitive and economical method It has been successfully used to obtain results which have conformed with the requirements of the Ministerial Decree (Segre 1929)

There is thus no uniformity in the methods adopted in different parts of the world for the bacteriological analysis of water There has, however, been a great

deal of standardization and uniformity in the methods and technique of the chemical analysis of water for various purposes. Such standardization in procedure is also manifest to a considerable extent in the field of bacteriology and biochemistry as applied to medicine. It is not, therefore, clear why such a uniformity in methods could not be achieved also in the bacteriological analysis of water. If the standard lactose broth has failed to be a satisfactory primary medium, it should be promptly replaced by a more suitable one that would ordinarily require no confirmation in routine procedure. The proposed parallel planting in standard lactose broth and brilliant green bile broth does not appear to us to be either necessary or desirable. It is neither economical nor time-saving. Ruchhoft et al (loc cit) mentioned three factors which determine the rates of multiplication of B coli and B aerogenes in standard lactose broth, viz, (1) the initial physiological condition of the strains which determines the relative length of the lag phase, (2) the relative growth rates of the strains in their logarithmic phase, and (3) the relative sensitivity of the strains involved to the products of metabolism. They also refer to the ascendancy of one of the strains taking place during the first 24 hours while undergoing incubation at 37°C. These authors have not, however, clearly stated if the same factors would not operate with equal force, on the growth and multiplication in the standard lactose broth of the anaerobic and aerobic spore-forming and non-sporulating lactose fermenters and other symbiotic forms that are often found in the waters under test. We think that the factors referred to will operate in the same way. If the three factors, it would be reasonable to expect that the process of ascendancy of organisms other than the B coli and B aerogenes are thus influenced by the same one type over the rest might account for the suppression of the few coliform bacteria that tests and in some cases also for the suppression of the many spurious positive presumptive might be present in a water but could not show up, owing to the rapid over-growth of the other organisms. In the absence of an inhibitory agent in the medium that would effectively restrain these spurious forms from the very commencement of an analysis on a given sample of water, a contingency such as the one mentioned above could not be avoided. It would, therefore, be interesting to have some data from the work on parallel planting procedures, advocated by the Committee on Standard Methods, regarding the number of samples or tubes giving acid and gas in brilliant green bile broth (confirmed for coli-aerogenes subsequently) which, though presumptively positive in the standard lactose broth, failed to confirm for coli-aerogenes.

A great deal of diversity in thought and procedure was thus evident from a careful study of the published literature on the subject. We, therefore, proceeded to compare the standard and non-standard media advocated in the 'Standard Methods' (A P H Association, 1933) with one another and with the bile salt lactose broth of MacConkey, which is the standard medium in British practice. The results of our experiments and our findings thereon will now be set forth in detail, under separate headings. The different media and technique described in this inquiry were prepared according to the formulæ and technique described by the respective sponsors and the samples of water were inoculated into tubes containing the media in suitable strengths in such a way that the resulting dilutions were in every case uniform and constant. We give the general methods adopted by us in some detail, so as to enable other workers to under-stand the exact procedure we followed in this work.

The water under test is added to MacConkey's bile salt lactose broth, the strength of the media and the quantities of water added being shown in Table I —

TABLE I

Details of cultures in MacConkey's bile salt lactose broth

Number of dilution	Strength of medium	Amount of medium in c c	Test water added in c c	Number of tubes inoculated
A	Bacto peptone 6 per cent Sodium taurocholate 1 5 per cent Lactose 1 5 , Neutral red 1 0 ,, pH 7 4–7 8 .	7	20	1
B	50 per cent of A	7	10	2
C	50 ,, ,, A	5	5	3
D	33 3 ,, ,, A	3 to 4	1	3
			0 1	3
			0 01	3
			0 001	3

The cultures (as indicated in Table I) are then incubated at 37°C and examined for the production of acid and gas at the end of 24 and 48 hours At the end of 24 hours, however, the tube with the second smallest amount of water showing acid and gas is selected (or the tube with the largest amount of water if no other is positive or even if none of the tubes are positive) Dilutions from these tubes are sub-cultured on MacConkey's bile salt lactose neutral red agar (We adopted a slightly different procedure to the one detailed above, in the case of samples submitted to the brilliant green bile broth and the standard lactose broth comparisons with MacConkey's bile salt lactose broth—*vide* Sections 4 and 5 *infra*) Usually a 5 mm loopful of the culture is transferred to 9 c c of sterile distilled water, well shaken and a 3 mm loopful from this dilution is plated out on MacConkey's bile salt lactose neutral red agar with an L-shaped bent-glass rod The plate is then incubated at 37°C for 24 hours at the end of which period six to ten discrete pink colonies from a sector of the plate are fished off into bile salt D-tubes These are incubated for 24 hours examined at the end of this period for acid and gas, and a 3 mm loopful from each of the positive cultures is transferred to the following differential media —

(*i*) Nutrient broth for motility and indole formation

(*ii*) Buffered glucose broth for the methyl-red and the Voges and Proskauer tests

(*iii*) Koser's synthetic citrate medium for citrate utilization test

(*iv*) Saccharose, dulcite, adonite and inulin sugars for fermentation reactions

(*v*) Gram's staining reaction, from a 24-hour agar slope culture

The sugar reactions are recorded after 48 hours' incubation at 37°C , motility and Gram's staining reaction at the end of 24 hours, indole production after 72 hours and the methyl-red and Voges and Proskauer tests after 4 days Koser's

citrate utilization is recorded at the end of 24, 48 and 72 hours   The six or ten organisms thus studied are then classified into *B coli, B œrogenes* or *intermediates* This classification has, in our experience, afforded a most convincing confirmation of the presumptive positive tests   If such a classification were not made many waters containing lactose-fermenting organisms of the *colon-œrogenes* group, derived from sources which are probably innocuous, would stand to be unjustly condemned   This would particularly be the case with tropical water-supplies This view has been held by Clemesha (1908, 1912*a* and *b*), Archibald (1917), Wood (1919), Cunningham and Raghavachari (1924), Lewis and Pittman (1928), Martin (1934), Rider (1928), Brewster (1929), Taylor and Martin (1927), Topley and Wilson (1930), Poe (1931), Tonney and Noble (1932), Hirst (*loc cit*), Hicks (1929), Burke-Gaffney (1932), Gray (1932), Bardsley (1934), Thresh *et al* (*loc cit*) and Perry (1929)   There is clearly a growing conviction that the *B œrogenes* and *intermediate* forms are of little or no sanitary significance as a test for dangerous fæcal pollution of water supplies   Gray (*loc cit*) who holds the view that the *œrogenes* forms are very frequently met with in fæces and urine, and so cannot be considered to be entirely without significance, still gives it as his considered opinion that, when they are present in a water, they merely indicate, at best, pollution of a remote kind   If the views set forth above, including the slightly variant one of Gray, were correct, the confirmatory technique followed in American practice is not necessarily specific for proving truly fæcal pollution   It is not unlikely that, under these conditions, a number of waters are being unjustly condemned   Further, as we had already stated in a previous paper (Raghavachan and Seetharama Iyer, *loc cit*) the use of a broth devoid of an inhibitory agent for preliminary enrichment, the confirmation of only one of the presumptive positive tubes by the staining and morphological characters *alone* of *only two* colonies suspected to belong to the *colon-œrogenes* group, and finally the confirmation obtained by passing these two organisms through the same standard lactose broth devoid of any inhibiting agent, would appear to our minds to detract considerably from the value of the American Standard Methods as a reliable test for the study of *fæcal* pollution of water-supplies

Further, we feel that the confirmatory technique of the American Standard Methods is open to the grave objection that only the tube showing gas in the smallest amount of inoculum is selected and confirmed   A positive finding for coliform organisms is no doubt specific but a negative finding in that one tube does not preclude the possibility of coliform bacilli being present in any or all of the larger volumes   This would be true only of the standard lactose broth, as the presumptive positives in that broth are not necessarily also positive for *colon-œrogenes* organisms   Under these conditions, some samples of water, which stand to be condemned, might be certified as potable, while some other samples might be condemned as unfit for drinking on the assumption that if the smallest volume failed to confirm the higher ones (10 c c), shall be deemed to have confirmed for *coli* group   This appears to be the '*implied meaning*' given to the interpretation outlined in the American Standard Methods (Ruchhoft, 1935)   We are not able to accept the '*implied meaning*' given to an unconfirmed positive finding   It cannot be considered accurate or reliable under all conditions

The newer parallel planting procedure introduced into the latest edition of the Standard Methods is apparently not meant for universal adoption in all cases, so it

is too early to judge of its comparative value and merits   There can be no doubt,
however, that the new procedure necessarily involves a greater amount of media,
labour, time and skill   If a simpler, yet more reliable and single medium were
available   neither the   standard   lactose broth test, nor the parallel planting
procedure can have any claim to remain a standard procedure, or part of one

3   The American standard lactose broth compared with MacConkey s bile salt lactose broth

The samples of water used in this inquiry were derived from different sources,
scattered over the whole of the Madras Presidency—an area of over 143 870 square
miles, with a coast line of about 1,700 miles   The annual rainfall in the area varies
from 150 inches on the west coast to about 25 inches on the east   These samples
are, therefore, to be considered representative of different climatic and geological
conditions and of seasonal variations and, consequently well suited for making
comparisons of the value of different media in respect of different types of water
Eighty-six such samples were examined in this comparison   Routine samples
arriving in the laboratory, packed in ice and kept as near freezing point as possible
during rail transit, were used for the tests, so that no conscious selection was made
of the samples   The standard lactose broth (pH 6 8) was made in accordance with
the formula and technique outlined in the 7th edition of the Standard Methods of
the American Public Health Association (1933)   The sample of water under test
was added to this broth in tubes in the same quantities as those used in the
MacConkey series

<div align="center">Table II</div>

Source of sample	Total number of samples	MacConkey's bile salt lactose broth		American standard lactose broth	
		Presumptive positives	Confirmed	Presumptive positives	Confirmed
A Wells	17	14	14	16	13
B Rivers	13	13	13	13	13
C Infiltration galleries	17	13	13	14	11
D Lakes and impounded surface waters	14	12	12	12	11
E Filtered waters (slow sand and rapid mech)	12	9	9	9	7
F Chlorinated waters (all sources)	13	6	6	10	5
Totals	86	67	67 (100%)	74	60 (81%)

Table II gives the total number of samples from different sources that were
examined   the number that gave a positive presumptive test by the two methods

after incubation at 37°C for 48 hours, and the number of these presumptive positives that were confirmed for the presence of the members of the *colon-œrogenes* group

Presumptive test—While only 67, or 78 per cent, of 86 samples gave acid and gas in one or more of the tubes in the MacConkey series, 74 samples or 86 per cent yielded a presumptive positive test in the standard lactose broth   The number of presumptive positives in the case of raw water samples was the same in both media   In the case of well waters—deep and shallow wells containing water of good, bad and indifferent qualities are included—14 were positive in MacConkey's bile salt lactose broth and 16 in the standard lactose broth   But with treated, i e , filtered and chlorinated or merely chlorinated supplies, the figures were 28 and 31 respectively for the two media   A scrutiny of the positive presumptive tests at the end of 24 and of 48 hours' incubation reveals the fact that a positive reaction was found in smaller quantities of the inoculum after 24 hours in lactose broth in 28 cases In 45 cases both media gave identical results, while in 13 cases the standard lactose broth was found to be less delicate than the MacConkey's bile salt lactose broth The readings taken at the end of 48 hours showed the corresponding figures to be 43, 38 and 13 respectively   It is not safe to institute a comparison on these lines, when we are dealing with two totally different types of media, the presumptive positives in one of which are not necessarily of the same significance as in the other , for, as is well known, the positives in the standard lactose broth are often spurious Assuming for purposes of argument that the two sets of results are comparable, we find that in at least 13 out of 86 samples the MacConkey's bile salt lactose broth was able to detect lactose-fermenting coliform organisms in smaller volumes of water than the standard lactose broth

Confirmatory tests—All the 67 samples which gave a positive reaction in the MacConkey's bile salt lactose broth confirmed for the presence of *colon-œrogenes* organisms, a confirmation of 100 per cent, while only 60 or 81 0 per cent of the 74 samples positive presumptively in the standard lactose broth, similarly confirmed The percentage of confirmations in MacConkey's bile salt lactose broth for each of the different sources was 100, while it varied widely for the different sources in the case of standard lactose broth   Thus, it was 96 per cent for raw waters, 81 5 per cent for well waters, 78 per cent for filtered and only 50 per cent for chlorinated supplies   Nearly 20 per cent of the presumptive positive tests on standard lactose broth were due to lactose fermenters outside the *coli* group and had to be confirmed before the initial finding could be rejected   These results may be compared with those of Levine (1920) on the analysis of 1,559 samples in the advanced sector of the American Expeditionary Forces at Dijon in France   His samples were derived from both treated and raw supplies   His figures for confirmations from standard lactose broth were 97 7 per cent for untreated and only 44 per cent for chlorinated samples

Tables III and IV may be read together   The results of our tests have been classified in these two tables on the basis of two different sets of reactions generally used in differentiating lactose fermenters of the *colon-œrogenes* group into *coli*, *œrogenes* and *intermediate* forms   The methyl-red and indole tests alone have been used in framing our Table II, while Koser's citrate utilization test and the indole test have been employed in Table III   This latter combination of tests has given a perfect correlation and is, therefore, considered by us to be very well suited for differentiation purposes —

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TABLE III

A comparison of the lactose fermenters isolated from the two series on the basis of the methyl-red and indole tests

Source of samples	Number of samples	MacConkey's bile salt lactose broth				American Standard lactose broth			
		Number of cultures	Coli	Æro	Inter	Number of cultures	Coli	Æro	Inter
A Wells	17	119	81	34	4	104	49	54	1
B Rivers	13	100	62	23	15	91	40	10	11
C Inf galleries	17	86	44	38	4	69	38	28	3
D Lakes etc	14	97	51	29	17	97	13	36	18
E Filtered	12	75	33	38	4	56	30	23	3
F Chlorinated	13	42	21	19	2	37	20	12	5
TOTALS	86	519	292	181	46	454	220	193	41
PERCENTAGES			56 2	34 9	8 9		48 4	12 5	9 1

TABLE IV

A comparison of the lactose fermenters isolated from the two series on the basis of Koser's citrate and indole tests

Source of samples	Number of samples	MacConkey's bile salt lactose broth				American Standard lactose broth			
		Number of cultures	Coli	Æro	Inter	Number of cultures	Coli	Æro	Inter
A Wells	17	119	80	37	2	104	48	56	0
B Rivers	13	100	63	34	3	91	40	49	2
C Inf galleries	17	86	43	39	4	69	36	29	4
D Lakes, etc	14	97	46	43	8	97	33	52	5
E Filtered	12	75	32	40	3	56	20	23	0
F Chlorinated	13	42	21	21	0	37	20	14	3
TOTALS	86	519	285	214	20	454	217	223	14
PERCENTAGES			54 9	41 2	3 9		47 8	49 1	3 1

Table III 56 2 per cent of the total number of lactose-fermenting organisms isolated from MacConkey's bile salt lactose broth and only 48 4 per cent of those from standard lactose broth proved to be true *B coli* The standard lactose broth showed more *cerogenes* than the other, while the number of *intermediate* forms was the same in both

Table IV The percentages for true *B coli* by this classification have not materially altered for the two media There is a difference, however, in the figures for *cerogenes*, but the relative proportions have not materially altered The increase under the 'cerogenes' is due to the fact, that by this scheme of classification the 'intermediates' of Table III have been assigned to one or other of the two groups, viz, *coli* or *cerogenes*

<div align="center">TABLE V</div>

Reconstituted from Table IV, dividing the sources of the samples into three main types

Source of samples	Number of samples	MacConkey's bile salt lactose broth					American standard lactose broth			
		Number of cultures	Coli	Æro	Inter		Number of cultures	Coli	Æro	Inter
			Per cent					Per cent		
Wells	17	119	67 2	31 1	1 7		104	46 1	53 9	0 0
Untreated raw water	27	197	55 3	39 1	5 6		188	42 6	53 7	3 7
Treated Filtered and/or chlorinated water	42	203	47 3	49 3	3 4		162	55 0	40 7	4 3

From the above comparison the percentage of true *B coli* isolations appears to be higher for the standard lactose broth in the case of filtered and chlorinated supplies It should be remembered, however, that 28 and 33 samples out of 42 in this series yielded presumptive positive tests with MacConkey's bile salt lactose and standard lactose broths respectively, while all the 28 from the former and only 23 from the latter confirmed for *colon-cerogenes* This would discount the finding of a higher percentage of true *B coli* in standard lactose broth, on treated waters In the case of the other two sources which represent the large majority of the water-supplies in this Presidency, MacConkey's bile salt lactose broth has revealed the presence of considerably larger numbers of true *coli* than the standard lactose broth

Summary and conclusions —Eighty-six samples of water from different sources, representative of different climatic and geological conditions and of seasonal variations obtaining in the Madras Presidency—an area of about 150,000 square

miles—have been studied, using the standard lactose broth advocated in the 7th edition of American Standard Methods (1933) and the MacConkey's bile salt lactose broth of the British standard procedure

I   The results of parallel tests on these samples of water, using the two broths as preliminary enrichment and selective media indicate that the MacConkey s bile salt lactose broth test is more sensitive and more satisfactory and reliable, requiring no subsequent confirmation at all

II   Either as a selective medium for the preliminary presumptive test or as an enrichment medium to be followed up by sub-culture for the study of discrete colonies and classification into *coli*, *aerogenes* and *intermediates*, the standard lactose broth cannot be considered suitable or satisfactory

III   Gas production in standard lactose broth particularly with treated supplies (partially or fully purified or chlorinated waters) cannot be relied upon as *an index of the presence of the colon-aerogenes* group of organisms   Confirmation has to be made except perhaps in the case of raw untreated waters, known to be polluted   Again, the confirmation usually made of only that tube showing gas in the smallest volume of the inoculum is open to the objection that a negative finding does not preclude the possibility of *coli* being present in the larger volumes   Unless all the gas positive tubes are confirmed this test cannot be considered conclusive or absolute   With MacConkey's bile salt lactose broth, every presumptive positive tube is invariably confirmed

IV   The percentage of true *B coli* revealed by preliminary enrichment and subsequent confirmation in standard lactose broth is distinctly lower than that in MacConkey's bile salt lactose broth   The latter medium is obviously, therefore, better suited for the stimulation of the true *B coli* during their lag and logarithmic phases

V   The presence of an indicator viz, neutral red, in the MacConkey s bile salt lactose broth serves to indicate the formation of acid—one of the two primary characteristics of lactose-fermenting coliform bacteria   The standard lactose broth is lacking in such an indicator

4   THE AMERICAN STANDARD LACTOSE BROTH COMPARED WITH THE BRILLIANT GREEN BILE BROTH (AMERICAN PUBLIC HEALTH ASSOCIATION)

Forty-five samples of water from different sources were submitted to test using the two broths for preliminary enrichment   The broths were prepared in accordance with the formulæ and technique laid down in the American Standard Methods—7th edition, 1933, pp 115–119 (*loc cit*)   The pH of the standard lactose broth was adjusted to 6 8 and that of the brilliant green bile broth to 7 4 The dye used was Brilliantgrün (Grubler)

The technique adopted in the preliminary enrichment process was the same as that recorded in the previous section   A slight deviation was, however, made in the initial stage of the confirmatory procedure   Instead of using a single 5-mm loopful of culture from the presumptive positive tube showing gas in the second smallest volume of the inoculum, we transferred a 1-mm loopful from each of the positive tubes in every set of samples after 24 hours' incubation at 37°C to

9 c c of sterile distilled water   From this tube of sterile distilled water, inoculated with material from each positive culture, we plated out a 3-mm loopful on MacConkey's bile salt lactose neutral red agar   The subsequent piocedures were the same as those detailed above   The idea underlying this innovation was that we felt we would avoid possible negative results that may be due to the omission of some positive tubes, and we also hoped to obtain a greater variety of lactose-fermenting coliform organisms when present   Further, it was hoped that by this method every presumptive positive tube would have been subjected to a collective confirmation   The results amply justified our expectations

The tabular statements given in Table VI follow the same general scheme as in the previous section —

TABLE VI

Source of samples	Number of samples	AMERICAN STANDARD LACTOSE BROTH		BRILLIANT GRFEN BILE BROTH	
		Presumptive positives	Confirmed	Presumptive positives	Confirmed
A Wells	10	10	9	10	9
B Rivers	6	6	6	6	6
C Infiltration galleries	9	6	5	7	5
D Lakes, etc	6	6	6	6	5
E Filtered	6	4	4	4	4
F Chlorinated	8	7	4	5	4
TOTALS	45	39	34	38	33
PERCENTAGES			87 2		86 8

Presumptive test —Thirty-nine or 86 6 per cent of the total number of samples in the standard lactose broth and 38 or 84 4 per cent in brilliant green bile broth gave positive presumptive reactions on preliminary enrichment at 37°C for 48 hours

Confirmatory test.—Thirty-four and thirty-three samples, i e , 87 2 and 86 8 per cent of the presumptive positives in standard lactose broth and brilliant green bile broth respectively, confirmed for the presence of *coli-ærogenes*   The results are practically identical for the two media   But the presumptive positives and confirmations for *coli-ærogenes* in MacConkey's bile salt lactose broth for the same 45 samples were 37 and 37 respectively (*vide* Section 5 *infra*)   A confirmation of 100 per cent was thus obtained from the MacConkey's bile salt lactose broth, as there were no false positives in that series

TABLE VII

A comparison of the lactose fermenters on the basis of Koser's citrate and indole tests.

Source of samples	Number of samples	AMERICAN STANDARD LACTOSE BROTH				BRILLIANT GREEN BILE BROTH			
		Number of cultures	Coli	Æro	Inter	Number of cultures	Coli	Æro	Inter
A Wells	10	75	28	43	4	80	47	29	4
B Rivers	6	49	14	32	3	53	27	22	4
C Infiltration galleries	9	40	23	16	1	38	12	23	3
D Lakes, etc	6	57	23	18	16	40	12	28	0
E Filtered	6	33	17	16	0	38	27	10	1
F Chlorinated	8	31	18	9	4	27	15	11	1
TOTALS	45	285	123	134	28	276	110	123	13
PERCENTAGES			43 2	47 0	9 8		50 7	44 6	4 7

It will be seen from Table VII that the brilliant green bile broth revealed a larger percentage of true *B coli* (50 7) than the standard lactose broth (43 2)   The true *B coli* confirmation for the MacConkey's bile salt lactose broth for the same set of samples was 55 2 per cent   The brilliant green bile broth would, therefore, appear to have a more selective action than the standard lactose broth in detecting the true *B coli*, but not to the same extent as the MacConkey's bile salt lactose broth

TABLE VIII

Reconstituted from Table VII, dividing the sources into three types

Source of samples	Number of samples	AMERICAN STANDARD LACTOSE BROTH					BRILLIANT GREEN BILE BROTH				
		Number of cultures	Percentage of				Number of cultures	Percentage of			
			Coli	Æro	Inter			Coli	Æro	Inter	
1 Wells	10	75	37 3	57 3	5 4		80	58 7	36 2	5 1	
2 Untreated raw waters	12	106	34 9	47 1	18 0		93	41 9	53 7	4 4	
3 Treated or/and chlorinated	23	104	55 8	39 5	4 7		103	52 4	42 7	4 9	

While the brilliant green bile broth was able to show a larger percentage of true *B coli* in untreated raw waters and in well waters, the standard lactose broth appeared to advantage in the case of treated and chlorinated supplies A similar finding was reached in the previous comparison of the standard lactose broth with the MacConkey's bile salt lactose broth The brilliant green bile broth does not, however, possess the feature of the MacConkey's bile salt lactose broth that all the presumptive positives confirmed

TABLE IX

Showing the average number of species of coli-ærogenes *isolated*

Source of samples	Number of samples	AVERAGE NUMBER OF SPECIES PRESENT IN	
		American standard lactose broth	Brilliant green bile broth
A Wells	10	3 5	3 7
B Rivers	6	4 1	5 0
C Infiltration galleries	9	2 2	2 2
D Lakes etc	6	3 5	2 3
E Filtered	6	2 7	4 0
F Chlorinated	8	3 6	2 6
TOTALS	45		
AVERAGES		3 3	3 4

Although no definite superiority of one medium over the other is manifest from the final total averages, brilliant green bile broth appears to be slightly the better of the two if the individual variation in each group is considered

Summary and conclusions —Forty-five samples from different sources were studied using the standard lactose broth and the brilliant green bile broth for preliminary enrichment

I   The results of the presumptive positive tests were more or less identical in both media

II   Although the number of presumptive positives that confirmed was also practically the same in both media, the brilliant green bile broth showed on the whole a larger number of true *B coli* than the standard lactose broth   The latter showed, however, a slightly larger number of true *B coli* in treated and chlorinated supplies, while the brilliant green bile broth showed more true *B coli* than the standard lactose broth in untreated and well water-supplies

III   No definite superiority was established for either medium in the matter of the numbers of species isolated

IV   There is obviously no advantage gained by the use of brilliant green bile broth in preference to the standard lactose broth in the matter of presumptive positive tests and for confirmations, stopping short of further classification into *coli*, *aerogenes* and *intermediates*

5   Brilliant green bile broth and MacConkey's bile salt lactose broth compared

As already indicated in the preceding section, this comparison relates to the same set of 45 samples used in the previous comparison   The media and technique employed were likewise the same as those already described —

Table X

Source of samples	Number of samples	MacConkey's bile salt lactose broth		Brilliant green bile broth	
		Presumptive positives	Confirmed	Presumptive positives	Confirmed
A Wells	10	9	9	10	9
B Rivers	6	6	6	6	6
C Infiltration galleries	9	6	6	7	5
D Lakes, etc	6	6	6	6	5
E Filtered	6	5	5	4	4
F Chlorinated	8	5	5	5	4
TOTALS	45	37	37 (100%)	38	33 (86 8%)

Presumptive test —Thirty-seven or 82 2 per cent of the total number of samples on MacConkey's bile salt lactose broth and 38 or 84 4 per cent on brilliant green bile broth gave positive findings in the presumptive test

Confirmatory test —While all the 37 samples confirmed in the case of the MacConkey's bile salt lactose broth (100 per cent), 5 samples or 13 2 per cent of the 38 presumptive positives in the brilliant green bile broth failed to confirm These samples include a sample from a well, one from an untreated raw supply and three from treated waters   The confirmations in brilliant green bile broth were 90 per cent for well waters, 91 7 per cent for untreated raw waters, and only 81 3 per cent for treated samples, while the MacConkey's bile salt lactose broth gave 100 per cent confirmations under each of the three heads

TABLE XI

A comparison of the two media on the basis of the delicacy of reactions

Source of samples	Number of samples	BRILLIANT GREEN BILE BROTH AS COMPARED WITH MacCONKEY'S BILE SALT LACTOSE BROTH					
		Was more delicate		Was equal to		Was less delicate	
		24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
A Wells	10	1	5	8	3	1	2
B Rivers	6	1	0	2	4	3	2
C Infiltration galleries	9	5	6	2	2	2	1
D Lakes, etc	6	0	1	3	3	3	2
E Filtered	6	2	1	1	2	3	3
F Chlorinated	8	1	2	3	4	4	2
TOTALS	45	10	15	19	18	16	12
PERCENTAGES		22 2	33 3	42 2	40 0	35 6	26 6

Table XI shows that the brilliant green bile broth revealed the presence of lactose-fermenters in smaller volumes of the inoculum after 24 hours' incubation at 37°C in 22 2 per cent of the samples   In 42 2 per cent of the cases, both media gave identical results, while in 35 6 per cent of cases, the MacConkey s bile salt lactose broth contained lactose fermenters in smaller volumes than the brilliant green bile broth   At the end of 48 hours' incubation, there was little or no change in the number under those giving 'identical results', while there was an increase of 50 per cent under ' more delicate ' and a reduction of about 12 per cent under ' less delicate '   It is generally conceded that the true *B coli* tend to grow rapidly in broth cultures during the first 24 hours of incubation, after which the *ærogenes* forms multiply and produce acid and gas   The readings taken at the end of 48 hours are, therefore, not quite as significant of fæcal pollution in water, as those taken at the end of 24 hours   Kingsbury (*loc cit*) has definitely adopted the 24 hours' reading as being sufficiently accurate for the assay of potable waters in the Federated Malay States   Our own experience with Madras waters supports this view, although in practice we base our reports on the 48 hours' readings   The figures given in Table XI would, therefore, go to show that, on the basis of 24 hours' readings, the MacConkey s bile salt lactose broth was more delicate than the brilliant green bile broth in 35 6 per cent of the samples tested   On the basis of the 48 hours' reading also, it was superior to the brilliant green bile broth in 26 6 per cent of cases

TABLE XII

A comparison of the lactose fermenters on the basis of the methyl-red and indole tests

Source of samples	Number of samples	MacConkey's bile salt lactose broth				Brilliant green bile broth			
		Number of cultures	Coli	Ero	Inter	Number of cultures	Coli	Ero	Inter
A Wells	10	90	52	32	6	80	49	29	2
B Rivers	6	55	35	15	5	53	25	23	5
C Infiltration galleries	9	48	25	19	4	38	11	23	4
D Lakes etc	6	58	30	21	7	40	12	28	0
E Filtered	6	48	31	15	2	38	27	7	4
F Chlorinated	8	36	15	19	2	27	15	11	1
TOTALS	45	335	188	121	26	276	139	121	16
PERCENTAGES			56 1	36 1	7 8		50 4	43 8	5 8

TABLE XIII

A comparison of the lactose fermenters on the basis of Koser's citrate and indole tests

Source of samples	Number of samples	MacConkey's Bile Salt Lactose Broth				Brilliant Green Bile Broth			
		Number of cultures	Coli	Æro	Inter	Number of cultures	Coli	Æro	Inter
A Wells	10	90	51	36	3	80	47	29	4
B Rivers	6	55	35	19	1	53	27	22	4
C Infiltration galleries	9	48	24	20	4	38	12	23	3
D Lakes, etc	6	58	30	27	1	40	12	28	0
E Filtered	6	48	30	16	2	38	27	10	1
F Chlorinated	8	36	15	21	0	27	15	11	1
TOTALS	45	335	185	139	11	276	140	123	13
PERCENTAGES			55 2	41 5	3 3		50 7	41 6	4 7

Tables XII and XIII may be considered together

Table XII 56 1 per cent of the lactose-fermenting coliform bacteria in MacConkey's bile salt lactose broth and 50 4 per cent of those in the brilliant green bile broth belonged to the true *B coli* type   Brilliant green bile broth showed a distinctly higher proportion of *aerogenes* types than the MacConkey's bile salt lactose broth   The number of true *B coli* from untreated raw water-supplies was considerably higher in the MacConkey's bile salt lactose broth than in the brilliant green bile broth, 57 5 per cent and 39 8 per cent respectively The corresponding figures for well waters were, however, 61 2 per cent for brilliant green bile broth and 57 8 per cent for MacConkey's bile salt lactose broth respectively, while for treated waters, the figures were 51 5 and 53 8 respectively   The general superiority of the MacConkey's bile salt lactose broth over the brilliant green bile broth is again manifest

Table XIII While the percentage confirmations for true *B coli* have remained practically the same as in Table XII, many of the *intermediate* forms have been assigned to one or other of the two groups *coli* or *aerogenes* by this classification

TABLE XIV

Showing the average number of species of coli-ærogenes *isolated*

Source of samples	AVERAGE NUMBER OF SPECIES PRESENT IN	
	MacConkey's bile salt lactose broth	Brilliant green bile broth
A Wells	7 0	3 7
B Rivers	6 6	5 0
C. Infiltration galleries	3 7	2 2
D Lakes, etc	6 0	2 3
E Filtered	6 0	4 0
F Chlorinated	4 4	2 6
AVERAGES	5 7	3 4

The comparison in Table XIV is very instructive   The technique of isolation and the confirmatory procedure followed in each case were identical   The number of species of lactose-fermenting organisms of the *colon-aerogenes* group, from each source, was much higher in the MacConkey's bile salt lactose broth cultures than in the brilliant green bile broth cultures   We were able to demonstrate the presence of about 45 per cent more species in the samples cultured in MacConkey's bile salt lactose broth than in the brilliant green bile broth   It has already been proved that the true *B coli* isolations were more numerous in the former test   It appears, therefore, reasonable to conclude that the MacConkey's bile salt lactose broth with a single inhibitory agent is definitely more selective in its action for the *colon-aerogenes* group in general and for the true *B coli* type in particular, than the brilliant green bile broth, which contains two inhibiting agents   The greater sensitivity of the MacConkey's bile salt lactose broth over the other media is thus established

Summary and conclusions —A comparison of the brilliant green bile broth with MacConkey's bile salt lactose broth was made in the analysis of 45 samples from different sources

I   The results of the presumptive positive tests with the two media were nearly identical

II   MacConkey's bile salt lactose broth was more sensitive and more delicate than the brilliant green bile broth in detecting lactose fermenters in smaller volumes of the inoculum

III   The confirmations for true *B   coli* were more numerous in the MacConkey's bile salt lactose broth than in the brilliant green bile broth

IV   The average number of species of the *coli-aerogenes* group detected in each sample by using MacConkey's bile salt lactose broth for preliminary enrichment was 5 7 as compared with only 3 4 for the brilliant green bile broth

V   MacConkey's bile salt lactose broth has, therefore, a greater selective action and is more sensitive in detecting *B   coli* in water than the brilliant green bile broth

6   THE AMERICAN STANDARD LACTOSE BROTH COMPARED WITH SALLE'S CRYSTAL-VIOLET BROTH

Salle (1930) outlined a complete system for the bacteriological examination of water, in which he adopted crystal-violet as the agent for suppressing spurious lactose fermenters   He showed that more crystal-violet could be used than brilliant green, without exerting any more toxic action on *B   coli*, and that a better chance, therefore, existed with crystal-violet for the suppression of the Gram-positive organisms which caused the spurious reactions, than when brilliant green was used   Accordingly he devised and perfected a medium which has since been included in the list of the non-standard media given in the latest edition of the American Standard Methods (*loc   cit*)

By using this medium, Salle maintained that the production of acid and gas in practically 100 per cent of the samples examined was due to members of the *colon-aerogenes* group, and that more clear-cut results were obtained owing to the elimination of interfering non-colon organisms   Stark and England (*loc   cit*), however, disagreed with Salle's findings   They carried out 340 tests on this medium using 164 cultures of *B   coli* and 7 of *B   aerogenes* isolated from the faeces of 19 healthy individuals   Samples of sterile water were also artificially infected with 48 hours' broth cultures of these organisms, in amounts approximating the numbers that would be present in contaminated waters   Of 164 cultures thus tested, 154 or 93 9 per cent failed to produce 10 per cent gas in 24 hours in the crystal-violet broth   Seventy or 42 7 per cent similarly failed in 48 hours After five days, however, all of them produced gas, showing that the inoculated organisms were not all killed but only inhibited by the dye   They, therefore, concluded that this medium should not be adopted as a standard test medium in routine water analysis

The above findings were based on the results of tests with cultures isolated from human faeces and sub-cultured in the laboratory   The samples of artificially

infected water would, of course, be at best a near approach to contaminated waters found in nature   Again, Stark and England relied on the production of 10 per cent gas for a positive test   The rigid application of this arbitrary criterion is fast losing ground and the American Public Health Association is now considering the question of relaxing this rule   For, organisms of the *colon-œrogenes* group have frequently been isolated from fermentation tubes showing very much less than 10 per cent of gas   This has also been our experience   We, therefore, decided to test the value of Salle's crystal-violet broth on a few samples of water, using as controls (*i*) the American standard lactose broth and (*ii*) MacConkey's bile salt lactose broth (*vide* Section 7)

The media and technique adopted by us were in accordance with those detailed by Salle for the crystal-violet broth and with those outlined in the American Standard Methods (*loc cit*) for the standard lactose broth   Forty samples from different sources, arriving at the laboratory in the usual course (packed in ice), were thus tested   No conscious selection of samples was made

TABLE XV

Source of samples	Number of samples	SALLE'S CRYSTAL VIOLET BROTH		AMERICAN STANDARD LACTOSE BROTH	
		Presumptive positives	Confirmed	Presumptive positives	Confirmed
A Wells	6	4	4	5	4
B Rivers	7	7	7	7	7
C Infiltration galleries	8	7	4	7	6
D Lakes, etc	8	5	4	5	5
E Filtered	6	3	2	5	3
F Chlorinated	5	1	1	2	1
TOTALS	40	27	22 (81 5%)	31	26 (83 9%)

It will be seen (Table XV) that 27 or 67 5 per cent of the samples in Salle's crystal-violet broth and 31 or 77 5 per cent of those in standard lactose broth yielded presumptive positive results   But only 22 or 81 5 per cent of the total number of such positives in the former and 26 or 83 9 per cent of the presumptive positives in the latter confirmed for *coli-œrogenes*   There is thus no advantage gained by the use of Salle's crystal-violet broth in preference to the standard lactose broth, for preliminary enrichment

TABLE XVI

A comparison of the lactose fermenters isolated from the two media on the basis of the Koser's citrate and indole tests

Source of samples	Number of samples	SALLE'S CRYSTAL VIOLET BROTH				AMERICAN STANDARD LACTOSE BROTH			
		Number of cultures	*Coli*	*Æro*	*Inter*	Number of cultures	*Coli*	*Æro*	*Inter*
A Wells	6	23	11	6	6	23	16	5	2
B Rivers	7	41	22	17	2	36	24	11	1
C Infiltration galleries	8	24	17	7	0	29	14	11	4
D Lakes, etc	8	22	8	10	4	28	14	11	3
E Filtered	6	12	7	5	0	18	12	6	0
F Chlorinated	5	5	2	1	2	5	2	3	0
TOTALS	40	127	67	46	14	139	82	47	10
PERCENTAGES			52 8	36 2	11 0		59 0	33 8	7 2

It will be observed from Table XVI that while the true *B coli* confirmation from the standard lactose broth cultures was 59 0 per cent of the total number of lactose fermenters isolated, only 52 9 per cent of those from Salle's crystal-violet broth belonged to the true *B coli* type   The latter medium would, however, appear to stimulate the growth of the true *B coli* forms in treated waters to a greater extent than the standard lactose broth, as shown by Table XVII —

TABLE XVII

Showing the percentage of true B coli confirmations in the two media, from the three types of waters

Type of source	PERCENTAGE OF TRUE *B coli* ISOLATED FROM	
	Salle's crystal violet broth	American standard lactose broth
1 Wells	48 0	69 9
2 Untreated raw waters	47 6	59 4
3 Treated supplies (filtered or/and chlorinated)	64 0	53 9

But this apparent advantage in favour of the Salle's crystal-violet broth cannot give it any claim for superiority, as in the routine confirmations for the *coli-aerogenes* group from the presumptive positive tests on samples of treated water, the standard lactose broth showed 71 per cent positive against 63 per cent for the Salle's crystal-violet broth

Summary and conclusions —Parallel tests on 40 samples of water from different sources were carried out using Salle's crystal-violet broth and the standard lactose broth

I   The standard lactose broth gave a greater number of positive presumptives and a slightly higher percentage of confirmations than Salle's crystal-violet broth

II   Salle's crystal-violet broth revealed the presence of fewer true *B coli* in the completed confirmations, on raw and well waters, and of a comparatively larger number of these organisms in the case of treated waters than the standard lactose broth

III   Salle's crystal-violet broth cannot be considered to be a suitable preliminary enrichment medium in routine practice for all types of water

7   SALLE'S CRYSTAL-VIOLET BROTH AND MacCONKEY'S BILE SALT LACTOSE BROTH COMPARED

We have examined 54 samples of water from different sources, using the two broths mentioned above   No conscious selection of the samples was made (Table XVIII) —

TABLE XVIII

Source of samples	Number of samples	SALLE'S CRYSTAL-VIOLET BROTH		MacCONKEY'S BILE SALT LACTOSE BROTH	
		Presumptive positives	Confirmed	Presumptive positives	Confirmed
A Wells	12	7	7	7	7
B Rivers	7	7	7	7	7
C Infiltration galleries	9	7	5	6	6
D Lakes, etc	11	8	7	8	8
E Filtered	9	6	5	6	6
F Chlorinated	6	1	1	1	1
TOTALS	54	36	32 (88 8%)	35	35 (100%)

The presumptive positive findings for the two series are more or less similar
The percentage of confirmations, however, was only 88 8 in Salle's crystal-violet
broth as compared with 100 in the MacConkey's bile salt lactose broth   Salle's
crystal-violet broth yielded 100 per cent confirmation in the case of well waters,
93 3 per cent in the case of untreated raw waters and *only* 78 6 per cent for treated
supplies, whereas all the presumptive positive samples from each source in
MacConkey's bile salt lactose broth confirmed for the presence of *coli-œrogenes*

TABLE XIX

Source of samples	Number of samples	SALLE'S CRYSTAL-VIOLET BROTH AS COMPARED WITH MACCONKEY'S BILE SALT LACTOSE BROTH					
		Was more delicate		Was equal to		Was less delicate	
		24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
A Wells	12	1	2	8	8	3	2
B Rivers	7	0	0	4	6	3	1
C Infiltration galleries	9	1	3	4	2	4	4
D Lakes, etc	11	1	4	5	6	5	1
E Filtered	9	1	4	6	4	2	1
F Chlorinated	6	0	0	6	6	0	0
TOTALS	54	4	13	33	32	17	9
PERCENTAGES		7 4	24 1	61 1	60 0	31 5	16 8

It will be seen from Table XIX that, at the end of 24 hours, Salle's crystal-
violet broth was more delicate in only 7 4 per cent of the samples, and less delicate
in 31 5 per cent of the samples than the MacConkey's bile salt lactose broth   In
the remaining 61 1 per cent of samples both media gave identical results   The
readings taken at the end of 48 hours were almost the same under 'equal'
while the 'more delicates' increased from 7 4 to 24 1 per cent—this was due,
as will be shown later, to the higher proportion of *œrogenes* isolated from
Salle's crystal-violet broth—and the 'less delicates' decreased by about 50 per
cent   A larger number of positive presumptive tubes was obtained from
the MacConkey series than from the Salle series   As confirmations are made
only from the 24-hour broth cultures the advantage definitely lies with the
MacConkey's bile salt lactose broth.

TABLE XX

A comparison of the lactose fermenters on the basis of the methyl-red and indole tests

Source of samples	Number of samples	SALLE'S CRYSTAL VIOLET BROTH				MacCONKEY'S BILE SALT LACTOSE BROTH			
		Number of cultures	*Coli*	*Æro*	*Inter*	Number of cultures	*Coli*	*Æro*	*Inter*
A Wells	12	42	21	12	9	42	36	5	1
B Rivers	7	41	21	18	2	39	24	8	7
C Infiltration galleries	9	29	17	12	0	33	20	12	1
D Lakes, etc	11	40	18	13	9	46	33	7	6
E Filtered	9	30	15	11	4	29	10	18	1
F Chlorinated	6	5	2	3	0	6	6	0	0
TOTALS	54	187	94	69	24	195	129	50	16
PERCENTAGES			50 2	36 9	12 9		66 2	25 6	8 2

TABLE XXI

A comparison of the lactose fermenters on the basis of Koser's citrate and indole tests

Source of samples	Number of samples	SALLE'S CRYSTAL VIOLET BROTH				MacCONKEY'S BILE SALT LACTOSE BROTH			
		Number of cultures	*Coli*	*Æro*	*Inter*	Number of cultures	*Coli*	*Æro*	*Inter*
A Wells	12	42	19	21	2	42	36	6	0
B Rivers	7	41	20	19	2	39	24	12	3
C Infiltration galleries	9	29	17	11	1	33	20	11	2
D Lakes, etc	11	40	18	16	6	46	26	17	3
E Filtered	9	30	15	14	1	29	10	19	0
F Chlorinated	6	5	2	3	0	6	6	0	0
TOTALS .	54	187	91	84	12	195	122	65	8
PERCENTAGES			48 7	44 9	6 4		62 6	33 3	4 1

Considering Tables XX and XXI together, it will be seen that the true *B coli* in MacConkey's bile salt lactose broth were more numerous than in Salle's crystal-violet broth

Table XX   66 2 per cent of the total number of *coli-œrogenes* isolated from the MacConkey's bile salt lactose broth and only 50 2 per cent of those from Salle's crystal-violet broth were true *B coli*

Table XXI   62 6 per cent from MacConkey's bile salt lactose broth and only 48 7 per cent from Salle's crystal-violet broth were true *B coli* by this scheme of classification   The figures for *B œrogenes* show a difference without, however, any appreciable alteration in the relative proportions   The increase under ' *œrogenes* ' noticed in this table is due to a re-assignment of the ' *intermediates* ' of the previous table into either *coli* or *œrogenes* by the adoption of the citrate and indole tests as the basis of classification

TABLE XXII.

Showing the percentage of confirmations for true B *coli in the two media, dividing the source of samples into three types*

	Type of source	PERCENTAGE OF TRUE *B coli* ISOLATED FROM	
		Salle's crystal violet broth	MacConkey's bile salt lactose broth
1	Wells	45 2	85 7
2	Untreated raw waters	47 0	58 7
3	Treated waters (filtered or/and chlorinated supplies)	53 0	53 0

Salle's crystal-violet broth was as selective and sensitive as the MacConkey s bile salt lactose broth in detecting true *B coli*, in treated supplies   It was, however, inferior to MacConkey's bile salt lactose broth in the case of wells and to a lesser extent in the case of untreated raw waters   The selective action noticed in this comparison for Salle's crystal-violet broth—for treated waters—was also demonstrated in the previous comparison with the standard lactose broth   But the fact that a confirmation of only 78 6 per cent of the presumptive positive tests was obtained with this medium for the treated supplies, as compared with 100 per cent confirmation in MacConkey's bile salt lactose broth, would appear to detract considerably from the value of Salle's crystal-violet broth as a suitable enrichment medium for routine use in water analysis

Summary and conclusions —Parallel tests on fifty-four samples of water from different sources have been carried out using Salle's crystal-violet broth and MacConkey's bile salt lactose broth respectively as the preliminary enrichment and selective media

Comparison indicates that .—

I   Salle's crystal-violet broth gave almost similar results to MacConkey's bile salt lactose broth in the presumptive positive tests , but the confirmations for the *colon-œrogenes* group of organisms were 88 8 per cent and 100 per cent respectively

II   Salle's crystal-violet broth gave fewer true *B coli* in the completed tests than the other medium in the case of raw and well waters, and the same number of these organisms as MacConkey's bile salt lactose broth, in the case of treated waters   But this last-named advantage is largely discounted by the fact that the percentage of presumptive positive tests on treated waters that confirmed for *colon-œrogenes* was only 78 6 per cent as compared with 100 per cent for MacConkey's bile salt lactose broth

III   Salle's crystal-violet broth cannot, therefore, be considered a suitable preliminary enrichment or selective medium for routine use on all types of water MacConkey's bile salt lactose broth, on the other hand, effectively inhibits spurious lactose fermenters, stimulates the growth of the *colon-œrogenes* group in all types of waters, and offers the required optimum conditions for the growth and easy detection of the *B coli* types of organisms present in the water under test, from whatever source derived

8   Dominick-Lauter's methylene-blue brom-cresol-purple broth and MacConkey's bile salt lactose broth compared

The results of the above comparison have been published in a previous paper (Raghavachari and Seetharama Iyer, *loc cit*)   Only the conclusions reached will, therefore, be given here with such further observations as may be needed for comparative purposes

' Parallel tests on 72 samples of water from different sources have been carried out, using MacConkey's bile salt lactose broth and Dominick-Lauter's methylene-blue brom-cresol-purple broth respectively, as the enrichment and selective media Comparison indicates that the MacConkey's bile salt lactose broth test is more sensitive and more reliable '

Thus, while 98 3 per cent of the presumptive positives in MacConkey's bile salt lactose broth confirmed for *colon-œrogenes* group, only 93 1 per cent confirmed from the Dominick-Lauter broth   This latter medium yielded three presumptive positives all of which confirmed, in a total of seven chlorinated samples examined, while the MacConkey's bile salt lactose broth showed only one presumptive positive, which also confirmed   To this extent then, the Dominick-Lauter broth would appear to be slightly superior to the MacConkey's bile salt lactose broth, viz , in the testing of chlorinated supplies   But it should be stated that the percentage of true *B coli* isolated from the chlorinated samples on the new medium was only 61 0 per cent while 100 per cent of those from the MacConkey's bile salt lactose broth were true *B coli*

' Both as a selective medium for the presumptive test for *coli* and as an enrichment medium to be followed up by sub-culture for the study of discrete colonies and classification into *coli, œrogenes* and *intermediates,* MacConkey s

bile salt lactose broth (1 5 per cent bile salt) retains its position as the most satisfactory medium in water bacteriology '

Dominick and Lauter would not, however, accept the position allotted by us to the MacConkey's bile salt lactose broth, and so asked for a supply of our bile salt for carrying out a series of comparative tests   Their request was complied with, but we have not been furnished with their results as yet[*]

9   The Eijkman's fermentation test at 46°C. compared with results in MacConkey's bile salt lactose broth

A brief review of the literature will be given together with the conclusions arrived at in the comparisons made at this Institute by Webster and Raghavachari (*loc cit*), and later by Webster (*loc cit*)

Eijkman (*loc cit*) produced evidence that in Holland very pure waters showed no organisms capable of fermenting dextrose at 16°C even when quantities of 300 c c were tested   On the other hand, with heavily polluted waters this test was positive in much smaller quantities   A number of investigators have since studied Eijkman's methods   Huss (*loc cit*) stated that the Eijkman test often failed in the case of waters containing nitrates, as these offered protection to the glucose in the medium from that form of attack by *B coli*, which results in the production of gas, but not at all from that which results in acid formation from glucose   He advocated buffering of the water samples under test with one per cent K_2HPO_4 in order to ensure positive results   Leiter (*loc cit*) reported very favourably on the test and considered it to be specific for pollution from warm-blooded animals Perry (*loc cit*) found the test very satisfactory in the routine analysis of water and of oysters   Smit (1928) showed that this test gave a most reliable index of the age of the infection with *B coli*, and therefore preferred this index to the usual one of *B coli* numbers obtained by the use of other enrichment media   Brown and Skinner (*loc cit*) reported that the test was not uniformly reliable and satisfactory Ruchhoft *et al* (*loc cit*) came to a similar conclusion   De Maghalhæs (*loc cit*) produced evidence to show that the test was useful in differentiating *B coli* from warm and cold-blooded animals   The former fermented glucose broth at 46°C, while the latter did not   Taylor and Goyle (*loc cit*), working with Rangoon waters, were favourably impressed by the significance of this test   Kingsbury (*loc cit*) considered the test to be of but limited value   Webster and Raghavachari (*loc cit*) found the test unreliable for Madras waters   Martin (*loc cit*) studied over 2,000 samples of untreated waters in the Federated Malay States, using the Eijkman test and the standard MacConkey's test simultaneously, and reported that the results of both the tests were very similar

Williams *et al* (*loc cit*) reported very favourable results by using a modification of Eijkman's medium, in which a lesser amount of carbohydrate was used   All true *B coli* were found to survive in this medium   Webster (*loc cit*) carried out a series of comparative experiments using MacConkey's bile salt lactose broth at

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[*] We have since received a brief note from Lauter stating that the results of a few parallel tests run by him have checked well with their own medium, and that they propose to carry out a more extended series of tests

37°C , MacConkey's bile salt lactose broth at 46°C , and Williams' modification of the Eijkman broth at 46°C   The results with MacConkey's bile salt lactose broth at 46°C were very irregular   Williams' modified broth at 46°C was found to be much less sensitive for the presumptive test than the MacConkey's bile salt lactose broth at 37°C   Although it largely eliminated *B œrogenes* and the *intermediates,* yet it frequently failed to demonstrate true *B coli*   De Graaf (*loc cit*) showed that the brand of peptone used in making the Eijkman broth had a profound influence on the results obtained, and that streptococci flourished more readily in that brand of peptone which inhibited the *B coli* by producing more acid Perry and Hajna (*loc cit*) stressed the value and importance of the Eijkman's test in routine water analysis and advocated the use of a buffered broth with only 0 3 per cent of glucose

10 DISCUSSION

We have attempted, in the preceding sections, to present a concise summary of the literature on the subject of differential media used in water analysis as well as a detailed review of the results of our own comparative study of the different media

It is evident that the MacConkey's bile salt lactose broth (using the commercial sodium taurocholate in 1 5 per cent concentration) has not been given a fair trial by American water bacteriologists and by others outside America, who follow the American Standard Methods   When it came to be realized that the American standard lactose broth did not function satisfactorily, various other liquid media containing dyes were advocated for preliminary enrichment and for sub-cultural purposes   Bile has also received attention from some of the leading workers, but in the majority of cases either whole fresh bile or dried ox-bile alone has been experimented with   In the few cases where bile salts have been tried, concentrations of a pure salt higher than 1 5 per cent have invariably been used   We are unable to gather from the literature the reason why the ordinary commercial bile salt has not found more general application   Thresh *et al* (*loc cit*) have suggested that the reason why bile salt lactose broth has not been adopted in American practice is the fact that nearly all the water-supplies in America are derived from more highly contaminated sources than the sources of water-supply in England   This reasoning would appear to suggest that the MacConkey's bile salt lactose broth would work well, *only* in the case of relatively pure waters   MacConkey's bile salt lactose broth, using commercial bile salt, has been used extensively and with uniform success in all the tropical possessions of the British Empire   The water-supplies in most of these countries are similar to those of tropical and sub-tropical America, and the nature and extent of pollution in some of the less civilized countries comprised in the British Empire may be extremely bad   We, therefore, remain unconvinced by the reason suggested by Thresh *et al* (*loc cit*)

The MacConkey's bile salt lactose broth, we have used in our work, has conformed always to the formula adopted by Clemesha (*loc cit*) in his classical research on tropical water bacteriology   The British Ministry of Health (1934) has recently issued a report (No 71) on the Bacteriological Examination of Water-supplies   This memorandum is to be ' regarded, as suggesting standards for a routine in water examinations, thus contributing to a greater degree of unanimity

of opinion on the hygienic quality of any of the diverse water-supplies that may be submitted to the test' We have noted that, in the above report, the MacConkey's bile salt lactose broth described at page 31 includes 1 per cent sodium chloride for the double strength broth This addition will apparently become the routine in British practice It is not, however, in accord with the formula given by MacConkey (1905), Clemesha (*loc cit*), Levine and Schœnlein (1930), Stocks and Carey (1932) or Thresh *et al* (*loc cit*) We have not up to the present added sodium chloride to our MacConkey's bile salt lactose broth, or MacConkey's bile salt lactose agar So far as we are able to ascertain, direct experiments do not appear to have been carried out by the Committee responsible for the report issued by the British Ministry of Health (*loc cit*), to see if the addition of the salt would render the medium less suitable for the estimation of the coliform bacteria in water MacConkey's bile salt lactose broth and agar, with the sodium chloride added, are reported to have been in use both in London and at Manchester and to have given excellent results The addition of sodium chloride to MacConkey's bile salt lactose broth and agar would appear to have been the result of a ' desire on the part of the Committee to bring these media into line with the others and to standardize the same ' (Wilson, 1935)

Two to ten thousand samples of water have been examined every year in the Public Health Section of the King Institute of Preventive Medicine since 1908 These have included a wide variety of types, at all seasons of the year Most of the sources have been sampled regularly at least twice a year Experimental filter installations under the direct laboratory control of the Institute have been sampled regularly and from ten to twenty such samples are included daily in the total number Thus, nearly 50 per cent of the samples tested have been derived from sources that were constantly under our supervision and control We have thus had exceptional opportunities of following from day to day the various stages of purification reached in the different types of filters experimented with, and incidentally of proving the excellence of the MacConkey's bile salt lactose broth, which accurately revealed the quality of the water in these various stages We were enabled from the results obtained to arrive at a correct judgment of the performance of each type of filter under test during the entire period of its run We may therefore, claim with confidence that our media are fully standardized and have always yielded uniformly good results

During the long period of over a quarter of a century that the MacConkey's bile salt lactose broth and agar have been in use in this laboratory, there have been very few occasions when the quality of the water as determined by our routine tests, failed to correlate with the sanitary quality expected from a knowledge of the source concerned Spurious lactose fermenters have never troubled us

Different brands and different supplies of the various ingredients comprised in the media have necessarily had to be used during these twenty-seven years The concentrations of ingredients alone have remained the same throughout Bile salt (the commercial sodium taurocholate) has been used in a concentration of 1 5 per cent in making the stock broth-'A' solution referred to in Table I After dilution with varying volumes of water as set forth in that table, the final concentration of the bile salt in the cultures would be 0 3 per cent to 0 4 per cent This is in

accord with the optimum concentrations which were found by Salter (*loc cit*) to give good results   The pH of our liquid medium has always been between 7 4 and 7 8   We have used neutral red as our indicator   Sterilization of the liquid medium is effected by the discontinuous method, viz , steaming in an Arnold sterilizer for three consecutive days

The standard lactose broth of the American Standard Methods (*loc cit*) contains no inhibitory agent and has a pH value of from 6 1 to 6 4   The limitations to its use have already been given at length   The brilliant green bile broth advocated for parallel planting contains two inhibiting agents and the percentage of bile errs, to our minds, on the side of excess   From the results of our comparative experiments recorded in this paper, it would appear that brilliant green bile broth as a preliminary enrichment medium unduly restrains a fair proportion of the coliform organisms, which are revealed by the use of MacConkey's bile salt lactose broth, containing a single inhibitory agent, viz , bile salt (1 5 per cent)   Either the higher concentration of the bile, or the addition of the dye or possibly a combination of both, would therefore appear to be responsible for the lower percentage of confirmations and for the fewer number of true *B coli*—also the fewer number of species of *coli-aerogenes*—amongst the lactose fermenters isolated from the brilliant green bile broth, as compared with the MacConkey's bile salt lactose broth   In any case, when the commercial bile salt alone in a concentration of 1 5 per cent gives excellent results on all counts, the necessity for a combination of two inhibiting agents to achieve the same end is not manifest   Again, when the MacConkey's bile salt lactose broth would serve as a suitable preliminary enrichment medium, requiring no subsequent confirmation of the presumptive positive tubes, the proposed parallel planting on standard lactose broth and brilliant green bile broth appears to be an avoidable waste of time, media and energy

Salle's crystal-violet broth, involving the use of a single inhibiting agent—a dye of the para-rosaniline series—has not functioned well in our hands and is found to be definitely much inferior to MacConkey's bile salt lactose broth for general routine work   It is perhaps also slightly inferior to the standard lactose broth as judged by the results of our comparative tests   If the success of this medium in routine work depends on the quality or brand of the dye used, that factor alone would constitute a serious limitation to its universal adoption   This argument applies to the Dominick-Lauter broth and in fact to every other liquid medium in which dyes are used for inhibition purposes

The use of chemicals, such as phenol, boric acid, telluric acid, etc , has also been advocated but does not appear to have found many adherents

In the light of what has been stated above, we are left with but a single selective enrichment medium, MacConkey's bile salt lactose broth, which has stood the test of time and experience all over the British Empire   Different workers scattered over the whole world, in tropical, sub-tropical and temperate climates, have reported uniformly excellent results with this medium, not only in research but also in daily routine work   The results following the use of this medium in water analysis have correlated well with the sanitary survey of the supplies concerned in the vast majority of cases

- In tropical water analysis laboratories, MacConkey's bile salt lactose broth has been used with uniformly excellent results in assessing the sanitary quality of

water-supplies, it has served well to distinguish between recent and remote pollution

Before proceeding to offer a few suggestions, we consider it proper to give a short account of the general plan of a co-operative study on different media that has been initiated by the American Public Health Association (1935) during the past few months   A full account of this plan is to be found on pp 134—137 of the Year Book for 1934–35 issued by the said Association as a supplement to their official organ, the *American Journal of Public Health,* **25**   2, 1935   A dozen laboratories in the United States and in Canada have already agreed to co-operate and more are expected to join in the study   The methods that will be used are those described by Butterfield for the evaluation of media   Essentially, this procedure consists in adding various dilutions of a standardized suspension of organisms of the *coli-œrogenes* group to a series of tubes of each of the different media and determining the gas production   Definite directions have been issued by the Committee and the media, which are standardized Difco products, have also been distributed   The following media will be thus studied   (*i*) Standard lactose broth, (*ii*) Buffered lactose broth, (*iii*) Stark's formate ricinoleate broth, (*iv*) Brilliant green bile broth, (*v*) Salle's crystal-violet broth, (*vi*) Ritter's fuchsin broth, and (*vii*) Dominick-Lauter broth   The media giving the best results on the basis of the above tests with pure cultures will be subsequently tested for their practical value in water analysis   The Committee responsible for organizing the above co-operative study invite constructive criticism from the members of the Association

It will be seen that MacConkey's bile salt lactose broth is not included in this comparative study   We, therefore suggest that the Committee should include this medium also in their list   All the eight media would then be subjected not only to a comparison on the Butterfield method, but also to a simultaneous comparison on the routine water analysis procedure   A co-operative study such as the one planned need not be confined to the members of the Association but bacteriologists all over the world, members or not, who are engaged in water analysis might be invited to take part in the study and in the final discussion and interpretation of results   We believe that an accurate standardization of methods suitable for use in all climates would then alone be possible

We feel that the time is now more than ripe for a revision of the standard procedures with a view to obtaining simplification and uniformity   The presumptive test that will be ultimately selected should be simple and yet sufficiently accurate for all practical purposes, when carried out by different observers and with different waters

GENERAL SUMMARY AND CONCLUSIONS

A study of the available literature on the various media and technique used in the bacteriological analysis of water reveals the fact that there has been no uniformity on the subject amongst the various workers engaged in this field of bacteriology   There has also been a considerable amount of diversity in thought and procedure   The results of one worker are not comparable with those of another in a different country   There has been, so far, no real co-ordinated attempt at instituting a comparison of the different media with one another and, with what

is considered the routine medium by each worker, on a given set of water samples A study recently inaugurated by the American Public Health Association on such a co-operative basis has unfortunately omitted to include MacConkey's bile salt lactose broth in the comparison proposed The Committee, however, feel that the insistence hitherto placed by them on the production of ten per cent gas in the broth tubes for a positive reaction is needless and perhaps also unjust for determining the presence of organisms of the *coli-ærogenes* group

Our present study of the different media started early in 1933 would, therefore, appear to have supplied the omission referred to above We have used MacConkey's bile salt lactose broth at this Institute since 1908 with uniformly excellent results and spurious positives have never troubled us We have had an almost uniform 100 per cent confirmation of the initial presumptive positive tests Our results have correlated well with the quality of the water-supplies as judged from a knowledge of the local sanitary conditions The bile salt used by us is the ordinary commercial sodium taurocholate obtained from different English firms at different times as and when required and a concentration of 1 5 per cent has always been used From the results of our study the following conclusions appear to be justifiable —

I The American standard lactose broth yielded a larger number of presumptive positive tests than the other media tested Out of a total of 171 samples tested on this medium in the three sets of comparisons 144 were presumptively positive but only 120 confirmed All the presumptive positives in MacConkey's medium confirmed Of the positive presumptives in the brilliant green bile broth, Salle's crystal-violet broth and Dominick-Lauter medium, 86 8 per cent, 83 9 per cent and 93 0 per cent respectively confirmed for the presence of organisms of the *coli-ærogenes* group

II Brilliant green bile broth, when used for preliminary enrichment was less selective and less sensitive than the MacConkey's bile salt lactose broth It was more or less similar to the standard lactose broth as regards presumptive positives and confirmations, but showed the presence of a larger number of the true *B coli* in the completed confirmations than the standard lactose broth

III Salle's crystal-violet broth gave fewer presumptive positives and fewer confirmations than the standard lactose broth, but gave a larger percentage of true *B coli* than the standard lactose broth in the completed confirmations on treated waters

IV Dominick-Lauter's methylene-blue brom cresol-purple broth, though less sensitive than MacConkey's bile salt lactose broth, was definitely superior to the other broths included in this study

V The Eijkman's test was found to be unreliable for Madras waters Williams' modification of the above test, for which more satisfactory results were claimed, was also tested by Webster at this Institute and found unsatisfactory

VI The outstanding fact revealed by this study is that MacConkey's bile salt lactose broth is the medium of choice in routine water bacteriology as (i) it is very sensitive and selective in its action, (ii) it stimulates the growth of the *coli-ærogenes* forms, particularly the former, during the first twenty-four hours, (iii) it is consequently best suited to reveal the presence of these organisms which, in the present state of our knowledge, constitute the most reliable indication of the sanitary

quality of waters, (*iv*) it enables an accurate estimate of the pollution present in a water to be made in twenty-four hours after a sample is submitted to the test without the necessity for any subsequent confirmation, and finally (*v*) it has been found to reveal the presence of true B coli (either alone or in conjunction with other forms) in over 85 per cent of the samples that were presumptively positive in 24 hours   The corresponding figures were 75 per cent for the Dominick-Lauter broth, 73 per cent for the brilliant green bile broth, 71 per cent for the standard lactose broth and only 66 6 per cent for the Salle's crystal-violet broth

VII   It is suggested that MacConkey's bile salt lactose broth be included in the list of media to be submitted to co-operative study by the American Public Health Association, a plan of which study has been outlined in their Year Book for 1934–35*

We desire to express our grateful thanks to Lieut.-Colonel H E Shortt, I M S, Director of the King Institute, for his valuable advice and criticism in writing up this paper and to Major W J Webster, M C, I M S, for much helpful advice during the progress of our comparative study and also in the revision of this paper before it was sent to the press in its final form

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* We have since learnt (Parr, 1935) that a standardized Difco MacConkey's medium has recently been issued to a number of co operating laboratories in America, evidently for a comparative study

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AN IMPROVED TECHNIQUE FOR THE ISOLATION OF ASCARIS EGGS FROM SOIL

BY

P A MAPLESTONE, D S O , M B , CH B , D T M ,

AND

P K MUKERJI, M B , M SC

(From the Helminthological Research Laboratory, School of Tropical Medicine, Calcutta)

ISOLATION of ascaris eggs from soil is an important matter in some aspects of the study of the epidemiology of this infection   A considerable amount of work on this subject has been done in America and the Caldwells (1928) mention a method of isolating the eggs from earth   Briefly, this consists of mixing the soil to be examined with antiformin solution which is said to free the eggs from the soil   Sugar solution of high specific gravity is then added to the mixture to bring the eggs to the surface of the liquid from whence they are removed by means of a small phial or wire loop

As far as we are aware the details of the method have never been published but it has been made use of by Spindler (1929) and Brown (1927) as well as by the Caldwells themselves, and more recently Winfield (1935) working in China mentions its use   In all these reports of the use of this technique there is no mention of its quantitative value, by this we mean the proportion of the total number of eggs in a given sample of soil one may expect to recover, so although there is ample evidence that ascaris eggs are recovered from soil by this method there is no information as to how many eggs are left behind , in other words what is the degree of efficiency of this method

We have been carrying out systematic soil examinations for the presence of ascaris eggs for a considerable time, and at first we followed the Caldwells' method as being the only one available, but we used saturated salt solution instead of sugar and collected the eggs by ' direct centrifugal flotation pushed to finality '   The meaning of this, according to Clayton Lane, the originator of this centrifugal technique, is that the tubes are subjected to repeated centrifugalization with the addition of a drop or two more of salt solution, as required, and the application of fresh cover-slips until no more eggs are recovered

It was decided to test the above method to see how many eggs out of an estimated number in soil were recovered by it

Soil samples were prepared as follows An emulsion of a stool containing ascaris eggs was made by thoroughly mixing it with water until it was about the consistency of thin gruel It was then passed through a fine wire sieve to remove all lumps and coarse undigested matter, and it was finally thoroughly shaken In this way as even a distribution as possible of eggs throughout the stool was made Egg counts by the ordinary Stoll technique were performed Two c c of this stool was mixed intimately with 10 c c of earth that was free of all worms and their eggs These mixtures were placed in small tin boxes with lids and laid aside for varying periods before being examined so as to allow the stool to become as intimately incorporated with the soil as possible, and thus reproduce natural conditions as closely as practicable In all the experiments detailed below 10 c c of soil and 2 c c of stool prepared in the above manner were used

In the first experiment two specimens that had been kept 23 and 24 days respectively after the soil and stool had been mixed were placed in conical glass vessels and 10 c c of 30 per cent antiformin added to each They were then energetically stirred at frequent intervals for about half an hour and 25 c c of saturated salt solution was added After standing for half an hour the surface of the liquid was thoroughly skimmed by means of a wire loop and in one case no eggs out of an estimated number of 6,600 and in the other two out of an estimate of 4,400 eggs were recovered The practically complete failure in these two preliminary trials was probably, to a large extent, due to the dilution of the salt solution by the antiformin so that it was not of sufficiently high specific gravity to cause the ascaris eggs to come to the surface, therefore the next series of experiments were performed in such a manner that this dilution was avoided

The soil was placed in small flat-bottomed glass vessels and 25 c c of 30 per cent antiformin added to each , these were stirred at frequent intervals, as before, and then allowed to stand for the varying periods shown in Table I The antiformin was drawn off with a pipette and examined by centrifugalization to make sure that no eggs had been removed The antiformin-soaked soil was now transferred to ' d c f ' tubes and saturated salt solution added , they were then subjected to ' direct centrifugal flotation to finality ' and the number of eggs recovered was noted These are given in Table I —

TABLE I

Number	Number of days soil was kept	Approx number of eggs added	Time in contact with antiformin (hours)	Number of eggs recovered	Percentage of total eggs recovered
1	24	4,400	24	14	0 32
2	24	4,400	24	8	0 18
3	24	4,400	3	0	
4	24	4,400	3	0	
5	1	6,200	3	1,560	25 1
6	1	6,200	3	400	6 45

From these few results it seems safe to assume that prolonged soaking with antiformin is little if at all better than soaking for only a few hours, and except in the case of freshly-mixed stools and earth the method is not of very great value

In the next series of experiments the soil was placed directly into d c f tubes and 10 c c of 30 per cent antiformin added and they were frequently stirred for an hour They were then spun in the centrifuge to throw down the eggs and the antiformin drawn off with a pipette Saturated salt solution was added and the tubes subjected to d c f to finality The results are given in Table II —

TABLE II

Number	Number of days soil was kept	Approx number of eggs added	Number of eggs recovered	Percentage of total eggs recovered
1	21	6,200	2,062	33 2
2	21	6,200	1,360	21 9
3	19	9,800	48	0 49
4	19	9,800	30	0 30
5	19	9,800	20	0 20
6	19	9,800	35	0 35
7	19	9,800	765	7 8
8	19	9,800	26	0 26
9	19	9 800	26	0 26
10	19	9,800	17	0 17

With the exception of experiments 5 and 6 of Table I and 1 and 2 of Table II which were all done using portions of the same stool and which was different from those used in the other experiments it will be seen that very few eggs were recovered It seems probable that there was some unusual condition of the stool used in these four experiments which permitted the eggs to become free more easily than normally On account of the small number of eggs recovered in the remaining twelve experiments in which other stools were used it was resolved to try and devise a better method

As the action of antiformin in liberating eggs from soil apparently depends on the presence of free chlorine which is only present in this compound in small amount, it was thought that if a greater quantity of the gas were available the results might be better Accordingly a series of experiments was carried out in which prepared soil was mixed with tap water and thoroughly stirred at frequent intervals for one to two hours After this, chlorine was bubbled through the mixture for ten minutes, after half an hour it was spun in the centrifuge, the water drawn off,

saturated salt solution added, and it was subjected to d c f to finality as in the other experiments   Eight experiments were performed in this way and as no eggs at all were recovered the method was not continued with, as it was apparently much less effective than antiformin

As chlorine gas alone was totally ineffective in freeing ascaris eggs from soil and as antiformin contains alkali in the form of caustic soda it seemed that the latter must be of importance, so in the next series of experiments prepared soil was placed in large test tubes and mixed with 10 c c of 2 13 per cent caustic soda solution, as this is the strength we use in the preparation of antiformin   The tubes were frequently stirred for a period of one to two hours exactly as in the previous experiments and at the end of that time freshly prepared chlorine was bubbled through the mixture for ten minutes, and it was found that the mixture had become acid   After standing for about half an hour the mixture was transferred to d c f tubes and spun in the centrifuge to throw down the soil and eggs , the supernatant fluid was then drawn off with a pipette, saturated salt solution added and the tubes subjected to d c f to finality   The total number of eggs recovered from each of twenty-one preparations in this way is shown in Table III —

TABLE III

	MOIST SOIL					DRY SOIL			
Number	Number of days soil kept	Approx number of eggs added	Number of eggs recovered	Percentage of eggs recovered	Number	Number of days soil kept	Approx number of eggs added	Number of eggs recovered	Percentage of eggs recovered
1	28	4,500	2,300	51 1	1	33	4,500	1,460	32 4
2	63	10,000	3,920	39 2	2	28	4,500	1,360	30 2
3	58	10,000	3,824	38 4	3	31	4,500	1,147	25 5
4	26	4,500	1,632	36 27	4	32	4,500	1,040	23 1
5	31	4,500	1,520	33 7	5	34	4,500	820	18 2
6	32	4,500	1,480	32 9	6	84	26,700	2,960	11 1
7	33	4,500	1,160	25 7	7	63	10,000	980	9 8
8	60	4,500	1,060	23 5	8	79	26,700	1,260	4 8
9	34	4,500	1,020	22 6	9	79	26,700	1,060	4 0
10	80	26,700	4,520	16 9	10	84	26,700	680	2 5
11					11	58	10,000	242	2 4

Comparison of the figures in Tables II and III shows that the latter method of recovering eggs is distinctly better   It will be noticed in Table III that soils that have become dry during storage do not, on the whole, produce as many eggs

as those samples that have remained moist throughout   This is almost certainly due to the fact, demonstrated by the Caldwells (*loc cit*), that ascaris eggs become rapidly destroyed by drying, and the degree of dryness will naturally affect the number of eggs recovered   We confirmed this observation by taking three samples of soil identical with those used in experiments 2 and 3 (moist soil) and experiment 11 (dry soil) of Table III, and which had not become dry during storage   These were exposed to direct sunlight and they became quite dry on the surface   One was exposed for one day and 10 2 per cent of the total eggs were recovered, and the other two which were exposed for two successive days to the sun produced only 0 14 and 0 05 per cent of eggs respectively   In another series, ten samples of soil that had been kept for periods ranging between four and twelve weeks and which had become dry, less than ten per cent of the total number of eggs were recovered in every case

On account of the labour involved in preparing a fresh solution of sodium hypochlorite on every occasion it was thought that it might be possible to prepare a stock solution which could be kept for use when required   This was done by passing chlorine through a 2 13 per cent solution of caustic soda   The solution thus made was kept in an ice chest, and even with this precaution, it was found ineffective in less than a fortnight as fewer than 0 5 per cent of the total number of 26,000 eggs were recovered from three soil samples in which it was used

The probable explanation of this failure is that the apparent action of chlorine in freeing eggs really depends on the subsequent formation of hypochlorous acid and nascent oxygen from sodium hypochlorite *, and that as the dilute aqueous solution of sodium hypochlorite is unstable it soon becomes inactive It is therefore necessary to prepare the reagent freshly on each occasion
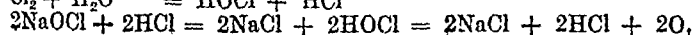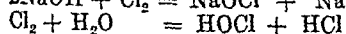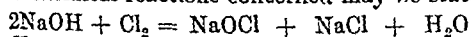
CONCLUSION

A new technique for the extraction of ascaris eggs from earth has been devised and an attempt made to compare it quantitatively with the method previously in use

The importance of drying of soil in the destruction of ascaris eggs has also been shown

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* The alkaline solution of sodium hypochlorite which occurs in antiformin has apparently slight action in freeing ascaris eggs from soil   Passing chlorine gas through tap water is also ineffective because of the small concentration of hypochlorous acid formed in this way, this in turn would give rise to only a small amount of nascent oxygen   But in the presence of sodium hydroxide a considerable concentration of sodium hypochlorite is obtained   When the reaction becomes acid, by the passage of chlorine through a solution of sodium hydroxide, a relatively high concentration of hypochlorous acid and hence of nascent oxygen is obtained   It is not known whether it is either the hypochlorous acid or nascent oxygen alone which is the important factor in freeing the eggs from the soil, or whether both these substances play a part in the reaction

The chemical reactions concerned may be stated as follows —

$$2NaOH + Cl_2 = NaOCl + NaCl + H_2O$$
$$Cl_2 + H_2O = HOCl + HCl$$
$$2NaOCl + 2HCl = 2NaCl + 2HOCl = 2NaCl + 2HCl + 2O,$$

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STUDIES IN THE SEROLOGY OF SYPHILIS

BY

CHARU CHANDRA BASU

AND

HEMENDRA NATH CHATTERJEE

(*From the Department of Pathology, Carmichael Medical College, Calcutta*)

THE main goal of serological tests for syphilis is that all syphilitic and only syphilitic sera should react in such a way as to give positive and unambiguous diagnosis At the present stage of our knowledge this is unattainable and still remains an ideal But much headway has been made and various points seem to have cleared up which have contributed to the increased sensitiveness of the reaction without any diminution in specificity

The work of the last two conferences of the League of Nations (1928, 1931) demonstrated the superiority of some of the flocculation tests over the modifications of the complement-fixation tests But later studies (Gilbert, 1930, Eagle, 1931, Basu and Chatterjee, 1934) showed that the complement-fixation tests employed did not fulfil the requirements of the newer knowledge on the subject When these modern conditions (Harrison, 1930, Gilbert, 1933, Basu and Chatterjee, *loc cit*) were fulfilled, the modifications of complement-fixation tests proved to be more sensitive than flocculation tests

In the endeavour to increase the sensitiveness of the complement-fixation tests and to measure quantitatively the amount of syphilitic ' reagin ' in the serum, the various directions in which the workers have proceeded may be summed up as follows —

(1) Increasing the sensitiveness of the antigen

(2) Varying the quantities of the different reagents and fluids, such as patient's serum, complement, antigen, amboceptor, etc , so as to get the optimum results

(3) Varying the temperature of incubation and the periods of reaction and finding out the ideal conditions

(4) Removal of substances which interfere with the sensitiveness of the reactions

INCREASING THE SENSITIVENESS OF THE REAGENTS

It is clear that the more sensitive the antigen, the lesser would be the amount of syphilitic antibody or ' reagin ' required to give a positive reaction

Of the various tissues, etc , which have been used as alcoholic extracts for antigens including human heart bovine heart, normal and syphilitic liver, syphilitic heart, as well as liver, kidney and brain of rabbits and guinea-pigs the most satisfactory have been the bovine and human hearts Of these two again, the bovine heart has given slightly better results in the hands of Kolmer and Trist (1922)

Crude extracts of heart muscle as well as the simple alcoholic cholesterol solutions have given far less satisfactory results than the two combined

The concensus of opinion now is that the addition of this sterol to the alcoholic extracts of heart greatly increases their antigenic sensitiveness in both complement-fixation and flocculation reactions

But a great deal of difference of opinion seems to exist as to the proper concentration of cholesterol in the antigens used for the complement-fixation tests

The outstanding contribution of recent years in this direction has been that of Eagle (1931, 1933) who super-saturated the alcoholic heart extract with cholesterol as well as with other sterols (corm germ sterol, sito-sterol, etc) A great increase in sensitiveness and a consequent detection of greater number of cases of lues is claimed thereby

In our previous work (Basu and Chatterjee, *loc cit*) we found a fair number of anti-complementary and non-specific reactions when using this antigen

The work of Levine (1932) also goes to disprove the claim of increased sensitiveness with the increase in amount of sterols Levine has shown that the amount of sterol required depends on the amount of lipoid present in the antigen and that concentration beyond a certain limit may actually diminish the sensitiveness of the antigen, and the phenomena observed follow a ' colloidal type of curve ' so that, after a certain concentration, the sensitiveness begins to decline according to general principles of colloidal reactions

Kolmer and Yagle (1934) have similarly obtained non-specific falsely positive reactions although they used a primary incubation of four hours (Eagle's method) as well as of 18 hours (Kolmer's method) They conclude that ' it is better to miss an occasional case of chronic latent syphilis than to run the risks of falsely positive reactions particularly in the routine work '

VARIATIONS IN THE DIFFERENT REAGENTS

Quantitative reactions are now preferred to purely qualitative ones and for this purpose any one of the different reagents, i e , serum complement, amboceptor, antigen, and sheep's corpuscles is used in different quantities, keeping the other reagents constant

In the present study we have used two methods side by side, viz , (1) that of Harrison in which different doses of complement are used and (2) a modification of the test in which the different dilutions of the sera are given

The sensitiveness of the Wassermann reaction has been found to be greatly increased by Fairbrother (1933) on using a serum dilution of 1 2 as compared with 1 5 in Harrison's test There appeared to be no reduction in specificity by this procedure

Wyler (1932) also obtained more sensitive results by using different gradations of serum

Kolmer (1929) after extensive comparative tests with the different methods comes to the conclusion that the serum dilution method is the best from the standpoint of economy, accuracy and ease of manipulation

From the theoretical standpoint also, this method seems to be the most scientific as it is the serum in which the syphilitic 'reagin' has been sought to be measured

The main disadvantage is the possible influence of natural hæmolysins which may be present in large doses of serum, this influence, however, is neutralized by methods described later on

VARIATIONS IN THE TIME AND TEMPERATURE OF REACTION

Jacobsthal (1910) first showed that the sensitiveness of the complement-fixation test could be greatly increased if the mixture of serum, antigen and complement was kept in the ice box prior to addition of sheep's cells

Since then various workers (Thomson and Boas, 1911, Ruediger, 1919, Kolmer, 1920, Kline, 1925, Eagle, 1933) have confirmed this phenomenon and there is now general acceptance of the fact that there is a significant increase in sensitiveness of complement-fixation with the preliminary incubation in cold But there seems to be a great difference of opinion amongst the workers as to the most suitable period for incubation

Some workers (Ruediger, *loc cit*, Kolmer, 1920, Kline, *loc cit*, Kilduffe, 1926) advocate 18 hours' incubation, while Eagle (1933) claims equally good results from a four hours' incubation, and others (Rice and Smith, 1933) again favour a two hours' incubation It seems possible that a particular time is suitable for a particular method (using a particular antigen)

Thus, in our observation with McIntosh and Fildes' antigen in the Harrison's method, some anti-complementary and doubtful reactions were obtained when the temperature of incubation was increased to 3 hours or more This will be evident from the following experiment —

TABLE I

		½ hour	1 hour	2 hours	3 hours	4 hours	18 hours
Negative sera	A	—	—	—	—	—	—
	B	—	—	—	—	—	—
	C	—	—	—	—	—	—
	D	—	—	—	—	—	—
	E	—	—	—	±	±	±
	F	—	—	—	—	±	±
Positive sera	P	+++	+++	+++	+++	Anti complementary	Anti complementary
	Q	+++	+++	+++	+++	+++	+++
	R	+++	+++	+++	+++	+++	+++
Antigen control tube		Hæmolysis	Hæmolysis	Hæmolysis	Hæmolysis	Hæmolysis	Anti complementary

The readings indicate the somewhat different results obtained with different periods of primary cold incubation followed by half-hour water-bath incubation at 38°C

For purpose of routine work we have, therefore, adopted one hour of cold incubation followed by a half-hour water-bath incubation although it is quite possible that greater periods of time may be suitable for other methods

REMOVAL OF SUBSTANCES WHICH INTERFERE WITH THE SENSITIVITY AND SPECIFICITY OF THE REACTIONS

(A) *Removal of the natural hæmolysins of the serum,*

(B) *Removal of the anti-complementary activity of the sera*

(A) The presence of natural anti-sheep hæmolysins is an important factor which has been observed to give false negative reactions, especially in those sera which contain small amounts of syphilitic antibody and large amounts of hæmolysins

Even when heated, about 85 per cent of the human sera contain some quantity of natural anti-sheep hæmolysin

The mechanism of their production is not yet known but they are acquired during the first year after birth

Unheated human sera contain different varieties of these natural hæmolysins

Some of these are thermolabile and are removed by heating to 55°C, others are comparatively thermostabile   The usual method that was used by us for their removal was   The addition of a drop of sheep's corpuscles to the unheated serum, and after thorough mixing and cold incubation for 15 minutes the separation of the sheep's cells by centrifuging and heating the serum at 55°C for half an hour

Recently Fairbrother and Peeney (1935) have observed that heating the sera at 58°C largely removes their natural hæmolysins but heating beyond this temperature has a tendency to diminish the complement-fixing antibodies as well

(B) *Anti-complementary action* —In our series of 561 cases only one case was found to give an anti-complementary reading in the tests used by us   It seems possible that heating at 55°C to 58°C is sufficient to remove the anti-complementary substances which are thermolabile

Some workers (Wyler, *loc cit*, Greval *et al*, 1929) use smaller doses of complement in the control tube   The idea is that weak and doubtful reactions can only be detected by smaller doses or complement in the control tubes   Presumably when small doses of complement are used in the control tubes these would naturally indicate the amount of anti-complementary action of sera and would eliminate a large number of doubtful reactions which are in reality caused by these anti-complementary bodies and not by the syphilitic 'reagin'   Greater quantities of complement would exhibit complete hæmolysis in the control tubes   But as complement is the most variable factor amongst all the reagents to omuch reliance can hardly be placed on the m h d of complement alone and in the small differences in the doses of complement (Harrison, 1930), especially in view of the fact that the behaviour of the complement does not always follow a mathematical rule

In fact this method as modified by Wyler (*loc cit*) was used in the 1931 conference of the League of Nations but was found to give the highest number of doubtful cases in the non-syphilitic sera amongst all the tests used

When the test is based upon the variations of complement the method of Browning and Mackenzie (1913 1924) though too elaborate for routine work seems to be ideal, the anti-complementary activity of each serum and the antigen are measured separately according to the number of units of complement fixed or absorbed by each, and the sum of these quantities of complement are added to the usual number of units as determined by the ordinary titration of complement

In our experience in a series of 95 cases a separate set of test-tubes was used containing 2 m h d of complement

In only one serum an incomplete inhibition of hæmolysis was observed, this specimen, however, gave negative reactions with all the four serological tests done by us

THE CLINICAL EVALUATION OF THE RESULTS

After incorporation of the above modifications and for the sake of evaluation of the results, different sera were examined by four different methods These were—

A   Complement-fixation tests—

(1) Harrison's method (1914, 1918)

(2) Serum dilution method

B   Flocculation reactions—

(3) Kahn's standard test (1926, 1928)

(4) Meinicke Klarungs reactions II (Meinicke, 1932a and b, Meinicke and Holthaus 1933a and b)

A   (1) *Harrison's method* as described by its author (1914 1918) was followed with the following modifications —

1   Natural hæmolysins were removed from the patients' sera

2   Every serum was heated for half an hour at 56°C

3   The primary incubation was done in the ice chest for one hour followed by a half-hour incubation in water-bath at 38°C before sensitized cells were added

4   Pooled sera of 2 to 5 guinea-pigs, kept overnight, were used for the complement

5   The four-tube test was done, the serum control tube containing 3 m h d and the front three tubes containing 3, 5 and 7 m h d of complement, respectively

A   (2) *Serum dilution method* —This test was done in a manner similar to Harrison's except that different dilutions of serum were used instead of that of the complement   The dilutions of serum used were 1 2, 1 4 and 1 8   The control tube contained serum in 1 2 dilution

A 3 m h d of complement was added to each tube, one unit of antigen suspension in the first three tubes and one volume of saline in the fourth tube   The rest of the procedure was exactly that of Harrison

The following symbols have been used in the interpretation of the results —

1   A strong reaction has been noted down as +++
2   A fair reaction has been noted down as   ++
3   A weak reaction has been noted down as   +
4   A doubtful reaction has been noted down as   ±
5   A negative reaction has been noted down as   —

Both + or ± readings denote that the serum is neither definitely positive nor definitely negative except in cases of treated syphilis when they might be regarded as indicating a positive reaction

The total number of cases in which a positive reaction was obtained was 243 Of these, the number of clinically diagnosed cases of syphilis, giving at the same time positive serological reactions, was 188—so that serological tests showed 55 more positive cases, or 40 per cent more, than clinical diagnosis alone

An analysis of these 188 clinically and serologically positive cases is given in Table II —

TABLE II

Test	Readings				
	+++	++	+	±	—
Harrison	87	43	27	14	17
Serum dilution	109	47	8	8	16
Kahn	82	37	19	11	39
M K R II	80	50	17	10	31

The above table may be further simplified by reducing the absolute figures in figures per cent and reducing the readings under three broad headings, viz , positive, negative and borderline (doubtful and weakly positive) cases

TABLE III

Test	Readings		
	+++ or ++	+ or ±	—
Harrison	130 (70%)	41 (21%)	17 (9%)
Serum dilution	156 (83%)	16 (8 5%)	16 (8 5%)
Kahn standard	119 (64%)	39 (16%)	39 (20%)
M K. R II	130 (70%)	27 (14%)	31 (16%)

The table shows that no one of these single tests could by itself diagnose all the cases   In order of sensitiveness the tests are put down as follows —

I　Serum dilution method

II　Harrison

III　M K R II

IV　Kahn standard

Thus the serum dilution method was unable to give any indication in 8 5 per cent cases, Harrison in 9 per cent cases, M .K R II in 16 per cent cases, Kahn in 20 per cent cases

A combination of the tests was able to give a serological diagnosis in all these cases

Taking into account the borderline and ambiguous cases, i e , those cases which gave + or ± results it would be found that the lowest figure occurred with serum dilution method   Of the two flocculation tests M K R II presented slightly better figures, by giving more definite reactions

RELATIVE AGREEMENT BETWEEN THE DIFFERENT TESTS

A　*Agreement between Harrison's method and other tests*

TABLE IV

Serum dilution	Kahn	M K R II
479 (86%)	373 (66%)	353 (63%)

B　*Agreement between serum dilution method and other tests*

TABLE V

Harrison	Kahn	M K R II
479 (86%)	373 (66%)	373 (66%)

C　*Agreement between Kahn standard and other tests*

TABLE VI

Harrison	Serum dilution	M K R II
373 (66%)	373 (66%)	458 (81%)

D   *Agreement between M K R II and other tests*

TABLE VII

Harrison	Serum dilution	M K R II
353 (63%)	373 (66%)	458 (81%)

The number of serologically and clinically diagnosed negative cases was 145

The clinically diagnosed negative cases which have given non-negative reactions are given   In all these cases the history was negative   Amongst the group will be found one case of psoriasis, 2 cases of epidemic dropsy and 2 cases of cancer in which syphilis was definitely excluded   There were also two cases of cerebro-spinal fever

TABLE VIII

Non-syphilitic cases giving non-specific reactions

Serial number of serum	Harrison	Serum	Kahn	M K R II	
6	—	±	—	—	Epidemic dropsy
10	—	—	±	—	Retinitis
11	—	-|	—	—	C S fever
27	—	±	—	+++	Cancer, stomach
98	±	±	—	—	Nephritis
33	+	—	—	±	Hypospadias
135	—	—	—	±	C S fever
156	±	—	—	—	Puerperal sepsis
158	±	—	—	—	Facial paralysis
166	—	—	±	—	Anæmia
277	±	—	—	—	Cancer
8,691	±	±	—	—	Psoriasis
8,793	±	—	—	±	Colitis
8,715	±	—	—	—	Old iritis
8,727	—	-|	—	—	Malaria with nephritis
8,794	—	—	—	±	Peritonitis

TABLE VIII—*concld*

Serial number of serum	Harrison	Serum	Kahn	M K R II	
8,834	—	—	—	±	Cancer
8,990	±	±	—	—	Keratitis
9,018	—	±	—	—	Sinus
9,039	—	—	—	+	Glaucoma, epidemic dropsy
9,040	—	—	—	±	Optic neuritis

Although the number of cases are too few for scientific generalization the writers have observed that, in the present series and in subsequent cases, the serum of epidemic dropsy and cancer cases gave a positive reaction with M K R II test The point, however, requires further investigation It would be observed that none of the cases have shown a definitely positive reaction (i e , +++ or ++) with the complement-fixation tests

POSITIVE CASES GIVING NEGATIVE REACTIONS

These cases have always been one of the great difficulties of the serology of syphilis

Leaving aside the nine treated cases of syphilis there remain another nine cases which gave absolutely negative reactions with all the tests

Of these cases two were of pregnancy with abortion Other workers have noted negative reactions after the delivery in pregnant cases Kolmer (1929) observes ' It would appear to be definitely established that Wassermann reaction in pregnant syphilitic women may become negative for a time after delivery and this is responsible for much confusion '

Of the remaining seven cases five were cases of neuro-syphilis and two of tertiary syphilis

The serology of neuro-syphilis present certain difficulties (Kolmer, 1929 , Meninger and Bromberg, 1935) and the question of the neurotropic strains of *Treponema pallida*, and the possible serological results following infection with them requires further study

The total number of neuro-syphilis cases was 25, and when this figure is taken into account it must be admitted that the positive diagnosis of neuro-syphilis cases is much less satisfactory than that of other cases

Kolmer (1929) sums up the whole question as follows ' At any rate negative blood reactions and even repeatedly negative reactions alone never rule out the possibility of a neuro-syphilitic infection since they may occur anywhere between 10 per cent and 30 per cent of cases showing positive spinal fluid reactions '

Thus, in our series 5 out of 25 cases of neuro-syphilis showed negative serum reactions

If we exclude these neuro-syphilitic sera then 2 out of 561 cases or less than 0 5 per cent have failed to be detected by the diagnostic methods

If, however, the neuro-syphilis cases are taken into consideration the percentage would go up to 1 5

When only the clinically diagnosed cases of syphilis are taken into account then the number of undiagnosed cases would be 3 5 per cent

On this question the work of Hoverson *et al* (1933, 1935) seems to break new ground   They have demonstrated the seasonal variations of known untreated syphilitic sera   Thus a positive serum may be negative owing to climatic and metereological conditions although the patient has not been receiving any treatment

This work further reduces the value of negative findings and emphasizes the importance of repeating the tests at different intervals

Similarly, Boas (1934) has twice observed positive serological reactions turning negative in the absence of any specific treatment   The Wassermann reaction was repeated several times but it remained consistently negative

REACTIONS IN OTHER DISEASES

The possible association of epidemic dropsy and cancer with positive serological tests especially M K R II has already been suggested

Malaria —Of the 39 cases associated with malarial fever 26 gave negative results, seven gave positive results in syphilitic cases and in the remaining six, in whom syphilis could not be excluded, doubtful reactions were obtained

Kala-azar —Of 5 cases 3 gave negative reactions and in the remaining two with syphilis positive reactions were obtained

SUMMARY

1   The sera of 561 cases have been studied by means of two modifications of complement-fixation tests and two flocculation tests

2   An endeavour has been made to modify the complement-fixation in the light of recent work on serology

3   No single test has been able to diagnose all the cases of clinically known syphilis , the combination of more than one test is recommended

4   Quantitative complement-fixation tests with variations in serum have given much more satisfactory results than those in which variations in complement are used

5   Judged from the point of view of sensitiveness the tests may be placed in the following order —

    1   Serum dilution method (complement-fixation)

    2   Harrison's method (complement-fixation)

    3   Meinicke Klarungs reaction II (flocculation)

    4   Kahn standard test (flocculation),

6 Meinicke Klarungs reaction II is more sensitive than Kahn but has a tendency to give more non-specific positive reactions in epidemic dropsy and cancer cases, a point which ought to be investigated

7 About 3 5 per cent cases of clinically known syphilis still remained undetected even when multiple tests were done Of this neuro-syphilis included 2 per cent and other varieties 1 5 per cent

8 Malaria and kala-azar do not seem to have any effect on the tests

Our best thanks are due to Dr N B Mondal, Dr N G Biswas and Mr J C Sur for the very great help we have received from them

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BLOOD GROUPS OF THE ANGAMI NAGA AND THE LUSHAI TRIBES

BY

CAPTAIN P N MITRA, M B , D T M ,

Formerly Teacher of Pathology, B W M School, Dibrugarh

THE Angami Naga and the Lushai tribes occupy certain of the hill ranges running between Assam and Burma   Politically they belong to Assam   They live in small villages in the midst of dense forests   Not being of commercial importance their hills are not easy of access

It was not possible to visit the tribes personally to determine their blood groups on the spot but Dr Das and Dr Chowdhury kindly undertook to collect specimens for me from members of each of the tribes   Blood was collected by pricking the finger and allowing a drop to run into a capillary tube which was immediately sealed in a flame and posted to me at Dibrugarh   Mino (1923) has found that red blood corpuscles, preserved in this way, remain perfectly agglutinable for a long time and Pijper (1932) has also successfully used this method for examining the blood groups of the Bushmen

GROUPING TECHNIQUE

The contents of a capillary tube were evacuated into a test-tube containing normal saline   After slight shaking so as to form a suspension of red blood corpuscles the supernatant suspension was pipetted off, leaving a small clot behind   The second tube was centrifuged and the supernatant fluid was pipetted off, leaving the red blood corpuscles in the bottom   Enough normal saline was now added to make an approximate 5 per cent suspension of the red blood cells   The further technique is the same as has recently been described by Mitra (1935)   Hæmolysed tubes were discarded

The results are given in Table I —

TABLE I

Name of tribe	Number examined	GROUPS IN PERCENTAGES				Biochemical race index
		A	B	AB	O	
Angami Naga	165	38 78	11 52	3 64	46 06	2 8
Lushai	141	44 68	16 31	6 38	32 63	2 3

As the various tribes are isolated from each other as well as from the people of the plains of Assam, it naturally is interesting to compare their blood groups with those of neighbouring people   Unfortunately, there is no record known to me of the blood groups of the Burmese or of any hill tribe of Assam   In Table II, however, their blood groups are compared with those of the people of the plains of Assam as worked out by Mitra (1933) and with the blood groups of the people of Hunan in South China, as worked out by Chi-pan (1924) —

TABLE II

Serial number	Name of tribe or race	GROUPS IN PERCENTAGES				Biochemical race index	p	q	r
		A	B	AB	O				
1	Angami Naga	38 78	11 52	3 64	46 06	2 8	24 1	7 9	67 9
2	Lushai	44 68	16 31	6 38	32 63	2 3	30 1	12 1	57 1
3	Chinese of Hunan	38 80	19 40	9 80	31 80	1 6	28 5	16 0	56 3
4	Assamese of the plains	24 55	32 55	9 25	33 65	0 8	18 6	23 7	58 0

It is seen that the Angami Naga and Lushai tribes (Nos 1 and 2) show a high percentage of A, a comparatively low percentage of B and a high biochemical race index—points which are characteristic of the Hunan type of blood group (No 3), and can therefore be said to be related to that blood group   They differ, however, from the Assamese of the plains (No 4) who show a low incidence of A, a high incidence of B and a low biochemical race index—points characteristic of the Indo-Manchurian type of blood group

CONCLUSION

The Angami Naga 'and Lushai hill tribes on the borders of Assam show blood groups corresponding to the Hunan type, whereas people of the plains of Assam belong to the Indo-Manchurian type

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FAVUS IN INDIA

BY

N C DEY, L M P , L T M ,

AND

P A MAPLESTONE, D S O , M B , CH B , D T M

(*From the Medical Mycology Inquiry financed by the Indian Research Fund Association*)

(*School of Tropical Medicine, Calcutta*)

Achorion schönleini (Lebert, 1845) Remak, 1845,* is the cause of favus in Europe but a few other fungi of the group *Hyphomycetes* causing this condition have been described and they have also been placed in the genus *Achorion*   These are *A quinckeanum* Zopf, 1890, *A gallinæ* (Mègnin) Sabouraud, 1910, and *A gypseum* Bodin, 1907   Though clinically they cause lesions like those of favus, morphologically none of them show any end organs resembling those of *A schönleini*

In China and Japan cultures have been taken from clinically typical favus cases, but they differ from *A schönleini*   Thus we find *A formoseum* Hasegawa, 1927, *Grubyella schönleini* var *mongolica* Hashimoto and Ota, 1927, and *Grubyella schönleini* var *castanea* Taniguchi, 1925   Recently Chen, Kurotchkin and Hu (1931) described four different types of fungus with downy cultures, from patients with favus in Peiping, but the description of these is inadequate for their identification

Source of material —Recently a patient aged 16 years from the district of Joypur, Rajputana, who had been suffering from a condition of 'dry pustules' on the head and neck for the last eleven years, came to the Calcutta School of Tropical Medicine   The disease first appeared as a single scutulum when the boy was four

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* Synonyms —
Oidium schönleini Lebert, 1845   *Oidium porriginis* Montagne
Oospora porriginis (Mont et Berk) Saccardo, 1886
Oidium (Achorion) schönleini Zopf, 1890
Grubyella schönleini Ota and Langeron 1923
Trichophyton schönleini Langeron and Milochevitch, 1930

years old and gradually the whole head had become affected and was covered by typical favus crusts of the usual sulphur-yellow colour (Plate XIX, figs 1 and 2) The hairs growing through the scutula were lustreless, but not broken and they were very sparse in the affected areas   On removal of the crusts there was an excoriated layer of skin covered by a thin layer of epithelium   The contiguous parts of the neck were also affected but the lesions were *very superficial and without* the characteristic appearance   The source of infection could be traced to the boy's mother who had suffered from a similar ailment since her childhood (as reported by her brother who brought this patient)   No one else in the house had the disease

Mycology —The crusts were found to be full of spores varying from 4μ to 6μ in diameter and short shreds of mycelia with rounded ends of the same breadth and of variable length   Some spores were budding in places and were irregular in shape   The mycelia were curved and comma-like or sinuous and were either branched or simple (Plate XX, fig 3)

Hairs, when treated with sodium sulphide solution, did not show any mycelia for a period of 3 to 4 hours except where the crusts were adherent   There was no air bubble adhering to the hair , this is said by Sabouraud to be characteristic At the end of 3 to 4 hours when lysis and disintegration of the hair cells had taken place, the mycelia appeared   Some were beaded but most of them were without any arthrospores such as the vegetating mycelia of the crust contained   On inoculation of either the crust or hair growth took place quickly and sub-cultures were made in different media to ascertain if the characters would vary under these conditions

Cultures were made on Sabouraud's maltose- and glucose-peptone agar, glucose agar, Czapek's synthetic agar, nutrient agar slope, semi-solid starch (4 per cent), glucose broth, inspissated serum, starch agar with and without peptone, Petroff's medium, dhâl broth, dhâl agar, potato medium, blood agar, whey agar, Dorset's egg medium, glycerine agar and liquid glucose-peptone   The organism was also inoculated on Sabouraud's maltose-peptone agar with the oxygen supply limited by a rubber cap on the mouth of the test-tube and also under partial and complete anaerobic conditions   The results are as follows —

Sabouraud's maltose-peptone agar —At the end of the first week, the growth is 1 25 cm in diameter, convex, raised, slightly cerebriform with white chalky powder spread uniformly over the growth   The margin shows fibres radiating from the growth forming a fine halo beyond which there is another lighter halo

At the end of the second week, the growth is about 2 cm in length, the colony becoming flattened in places though the character is still cerebriform   The margin of the growth shows a fringed appearance

At the end of the third week, mycelia are climbing along the side of the test-tube   The growth is 2 5 cm in length and is otherwise unaltered but submerged growth on the both ends is now taking place (Plate XXI, fig 5)

At the end of the fourth week, the growth is losing the white powder and becoming smooth   The medium has become brown especially under and round the growth   The colouring of the medium follows the smoothing of the growth which takes place after a variable time in different specimens and *also on drying of the medium*

In a flask, where the space for growth is large, the fungus grows to a size of 3 cm to 3 5 cm in diameter in 3 weeks with a white chalky crust   There is always a fibrillar, fringed margin heralding spread of the growth   Secondary growth at the periphery appears on the fourth week or later   In the flask the primary growth does not become any bigger but secondary growth takes place to a slight extent   Cultures when old always gradually lose the white chalky character in Sabouraud's medium (Plate XXII, figs 11 and 12)

Sabouraud's glucose-peptone agar —The growth in this medium is more rapid than in any other and consequently although cerebriform it only appears as a wrinkled and folded thick membrane   There was no brown coloration of the medium or of the growth

The growths on the fourth day after inoculation vary in different tubes from 0 75 cm to 1 cm in diameter   They are raised, convex and cerebriform with snow-white chalky powder uniformly distributed on the surface   There are irregular pits on the surface of the growth like favic scutula

At the end of the first week the growths are from 1 25 cm to 1 5 cm in diameter and are raised, with superficial radial furrows in some places   The marginal part of the growth shows radiating fibres intimately mixed with the surface of the medium, visible in transmitted light   These fibres form a halo about 0 25 cm in width round the growth and blend with the medium   The central growth is white as it is covered by a uniform layer of fine powder

At the end of the second week, the character of the growth is similar to that of the first week but it is more elevated and cerebriform in the centre, the pits are more marked and the margin of the growth shows further fibrillar extension lengthwise along the slope   The area of growth including the fibres is 3 cm by 1 5 cm (breadth of the slope) but the main cerebriform growth is about 2 cm in length

At the end of the third week and later, the character of the growth is the same   The centre is cerebriform and about 2 25 cm in length   Beyond this area there are fibres extending about 1 25 cm above and below the central growth   These fibres are mixed up with the medium and go on growing until they reach the ends of the culture medium   There is no coloration of the medium   The growth is brown in colour in places where it has lost the powder   This loss begins about the fourth week and the brown colour becomes deeper and powder is totally lost in 4 months or so and the growth is then completely glabrous

Glucose agar —At the end of the first week, the growth is raised, convex and irregularly circular, about 1 25 cm in diameter and covered uniformly by a comparatively thick coat of white powder   There are five or six furrows radiating from the centre

At the end of the second week, the character of the growth is much the same   The area is about 1 5 cm by 2 5 cm in length and mycelia are growing on the sides of the test-tube   White powder is still more evident on the surface of the growth and the radiating furrows are deeper

At the end of the third week, the growth has spread lengthwise for about 4 cm covering the slope completely   The centre is cerebriform and the furrows extend in a radiating manner over the surface (Plate XXI, fig 6)

At the end of the fourth week in one tube the medium, which is drying, has become orange red or salmon pink and the culture is more wrinkled on account of drying   Another tube, which is not so dry, does not show staining of the medium

Synthetic agar (modified Czapek's) —At the end of the first week the growth is wholly submerged in the medium except at the site of inoculation where the inoculated mass is above the surface   A very fine powder is spread on the surface and the submerged root structures are arranged radially and have gone to a considerable depth   The area of growth is 0·75 cm in diameter

At the end of the second week, the growth is about 1 25 cm in diameter and the character is not changed except that the roots are found to be deeper   This is best seen by transmitted light

At the end of the third week, the character of the growth is practically unaltered as there has been little further growth

At the end of the fourth week, the growth is 2 5 cm in length, and at the site of inoculation is raised above the surface of the medium and is about 0 20 cm in diameter.

Nutrient agar —At the end of the first week, the growth is 1 25 cm in diameter and is flat on the surface of the slope and is covered by yellowish-white down

At the end of the second week, the growth is more or less circular, 2 0 cm in diameter, the character being unaltered

At the end of the third week, the growth is spreading lengthwise along the slope for about 2 5 cm and is covered with fine brown down

At the end of the fourth week, the growth is 3 0 cm in length   It is flat and uniformly spread on the surface of the medium and is covered by fine brownish down

At the end of the sixth week, the tubes show velvety down intimately blended with the surface of the medium and almost covering the whole length of the slope (4 cm)

Semi-solid starch (4 per cent starch) —At the end of the first week, the growth floating on the surface of the medium is 0 75 cm in diameter with roots hanging down for about 0 25 cm

At the end of the second week, the growth is 1 25 cm in diameter with more roots developed   The growth is scum-like on the surface, has a cartilaginous feel and is attached to the side of the test-tube

At the end of the third week, the growth is 1 5 cm in diameter otherwise it is unchanged

At the end of the fourth week, as the medium is drying, there is a gap between the culture and the medium on one side, on the other side it has dropped and is lying on the surface of the medium, so that it appears obliquely placed

Glucose broth —On a sub-culture in glucose broth from a growth on Sabouraud's medium the glabrous or waxy parts generally settle down in the medium and the parts of the inoculated mass covered with white down or powder generally float on the surface   The parts that remain deep in the medium show a flaky, puff-like growth round the inoculated mass   The floating mass always shows a cerebriform growth from which roots hang down in the medium

After the inoculation in this medium some of the masses sank and one remained floating on the surface

At the end of the first week, there were woolly puff-like masses in the medium varying from 0 25 cm to 1 cm or more in diameter

The floating mass is cerebriform showing a somewhat honeycomb appearance and the size is about 1 cm in diameter with very fine pellicles growing and hanging down

At the end of the second week, flaky masses are numerous throughout the medium   They are more puffy than in the previous week and have a granular mass in the centre

The floating mass is now about 1 5 cm in diameter and knob-like with honeycomb or cerebriform appearance and a brownish-white colour

At the end of the third week, the flaky masses are unchanged   The floating mass is wrinkled, cerebriform, knob-like with a honeycomb appearance and occupying the whole of the diameter of the test-tube   The growth is folded on itself in a double layer as there is no space in the test-tube to grow further

The type of growth of the fourth week is like that of the third

Inspissated serum —At the end of the first week, the growth is about 1 cm in diameter   The medium is depressed in the centre, appears as if it is being lysed or liquefied at this spot and it is covered by white, thick, powdery masses

At the end of the second week, the growth is about 1 5 cm in diameter   The medium is more depressed in the centre and there is a light greyish-black zone of pigmentation around the central growth in which there are rough granular areas

At the end of the third week, the character of the growth is the same as in the previous week, except that the black zone round the growth is deeper than before There are rough, button-shaped masses 0 5 cm in diameter on the centre of the growth, and it is losing its downy covering

In the middle of the fourth week the growth is uniformly glazed in appearance due to fluid elaborated by the fungus from the medium

At the end of the fourth week, the growth is 2 cm in length and of a glabrous, faviform type and there is fluid at the bottom of the tube (Plate XXI, fig 7)

At the end of the sixth week, the slope at the site of the growth is completely liquefied and the liquid has settled at the bottom of the tube   The growth at this time shows pigmented and unpigmented portions mixed together , it is glabrous and corrugated

Starch agar with peptone —This medium consists of 4 per cent starch with 1 per cent peptone and 2 per cent agar in slopes

The growth at the end of the first week is 0 75 cm in diameter and is roughly circular   The central part is chalky and white, the margin glabrous and submerged

The growth at the end of the second week is 1 25 cm in diameter and roughly circular   The peripheral third shows a glabrous submerged growth   By transmitted light the glabrous part shows zones of varying density , of these the most peripheral is the lightest and they become gradually darker towards the centre

The peripheral part is submerged and is wax-yellow or cream-coloured and irregular in outline The roots have grown about 2 to 3 mm into the medium The piece of culture inoculated in the centre is glabrous and cream-coloured without a chalky crust

At the end of the third week, the growth is roughly oval and is 2 cm by 1 5 cm in area and the character is exactly the same as in the previous week The central part is chalky and is 1 cm in diameter In the peripheral part the growth is submerged and appears glabrous as there is no chalky crust over it

At the end of the fourth week, the growth is of the same appearance as in the third but it has increased in size to 2 75 cm by 1 75 cm

Starch agar without peptone (4 per cent starch and 2 per cent agar) —There was no visible growth till the third week

At the end of the third week there is very slight growth in both the tubes inoculated It is 0 8 cm in diameter, and is submerged in the medium appearing as a faint halo round the site of inoculation

At the end of the fourth week, the growth is 1 cm in diameter, no other change being noted

Petroff's medium (with gentian violet 1 in 1,000) —At the end of the first week a raised growth about 0 30 cm in diameter appears It is convex and convoluted though in a miniature form, yellowish-brown in colour, covered in places by very fine brownish-white powder intimately adherent to the growth

At the end of the second week, the character of the growth is the same as in the previous week except that it has increased slightly in diameter

At the end of the third week it is about 1 cm in diameter and though irregular in outline it is roughly circular and it is uniformly covered by a brownish-white powder

At the end of the fourth week, the area of the growth is slightly increased , it is cerebriform and there are some shallow pits on the surface The powder covering the centre of the growth is now yellowish-brown and the margin is brownish-white

Dhâl broth —At the end of the first week, there are two kinds of growth similar to those seen in glucose broth The inoculation that sank in the medium shows a puffy growth surrounding it This varied from 5 mm to 1 25 cm in diameter There were about four or five masses of that size and some smaller flakes

On the surface of the liquid there is a cerebriform growth floating as a knob-like honeycombed mass The area is about 1 cm in diameter and there are hanging roots though they are very short

At the end of the second week, the growth is much the same as in the previous week, but the flakes are more numerous The floating cerebriform mass is about 1 5 cm in diameter covering the whole of the surface The medium is coloured pink and the roots are a little longer

At the end of the third week, the puffy flake-like growth is unchanged but the surface growth is wrinkled and thrown into folds though the character is unaltered The medium is coloured darker pink with an orange tint

At the end of the fourth week, the surface growth is more wrinkled, the growth in the medium is unchanged and the medium itself is still darker, being now definitely orange

Dhâl agar —At the end of the first week, the growth is about 1 cm in diameter, cerebriform, slightly raised above the surface of the medium with irregular but superficial radiating furrows The growth is uniformly covered with a fine chalky crust intimately blended with the growth

At the end of the second week the growth is 1 5 cm in diameter, more convoluted superficially and covered uniformly with a chalky-white crust adherent to its surface The medium is coloured light pink

At the end of the third week, the growth has spread to 2 25 cm in length and the medium is a little more deeply coloured Other characters are the same as in the previous week, and no further change was noticed although the growths were frequently examined for more than six weeks (Plate XXI, fig 8)

Potato medium —At the end of the first week, the size of the growth is 0 5 cm in diameter, raised, cerebriform and almost completely covered by fine white granules The growth is raised about 0 3 cm above the surface of the medium

At the end of the second week, the growth is 1 25 cm in diameter, raised, superficially convoluted, covered by fine white powder and around the margin there is a greyish-black zone

At the end of the third week, the character of the growth is the same except that it is a little more chalky and drier than it was in the previous week and it is now 1 4 cm in diameter

In the fourth week the growth is more definitely chalky and cerebriform in appearance The zone of pigmentation round the growth is almost black It is about 1 5 cm in diameter

Blood agar —At the end of the first week there is a knob-like cerebriform growth 0 75 cm in diameter It is uniformly covered with white chalky powder and around it there is a submerged portion, greenish in colour The whole growth is 1 cm in diameter with a faint surrounding zone of hæmolysis

At the end of the second week, the growth is 1 5 cm in diameter, the central portion being 1 25 cm The character of the growth is unchanged except that the powder on the surface is thinner

At the end of the third week, the growth is 2 cm in diameter, the centre of the growth is more wrinkled, cracked, slightly depressed and glabrous from loss of the powder Beyond the central part, the powder that was white in the previous week has become greenish in colour The peripheral margin of the growth is faviform, glabrous and is slightly raised (Plate XXI, fig 9)

At the end of the fourth week, the character of the growth is unchanged except that the greenish powder on the surface is thinner The hæmolysis has extended throughout except to the two ends of the slope

Whey agar —At the end of the first week, there is a small central mass at the site of inoculation about 0 20 cm in diameter and elevated above the surface of the medium Around this there is a growth 1 cm in diameter which is largely submerged in the medium and distinctly visible by transmitted light.

At the end of the second week, the growth is smooth, submerged and blended with the surface and is 1 5 cm in diameter   The centre is covered with fine white powder and is slightly acuminate

At the end of the third week, the growth has spread lengthwise and very fine velvety powder is appearing on the smooth surface   The whole surface of the slope is covered and the growth appears moist

At the end of the fourth week, the growth is not much changed   The surface appears to be drier, and the size of the growth is 1 5 cm by 3 cm when examined against the light

Dorset's egg medium —At the end of the first week, the growth is 1 25 cm in diameter with six furrows radiating from the centre with geometrical accuracy

At the end of the second week, the growth is 1 75 cm in diameter   The centre is faviform, glabrous and finely folded, and around this there are furrows and folds wrinkled like a muslin frill

At the end of the third week, the wrinkles and furrows are more marked and the growth is becoming heaped up in the centre and upper end, it is thus convex in outline and appears drier than before   It is oval in shape, 2 5 cm by 2 cm in area and brown in colour

At the end of the fourth week, the central and upper parts of the growth show a fine honeycomb appearance of a dry type and are more raised and convex as well as being darker coloured (Plate XXI, fig 10)

At the end of the sixth week the upper portion is rougher and the honeycomb appearance is more prominent and it is also more raised and convex than in the fourth week   Later the whole growth developed a honeycomb-like series of fine crypts   The area of the growth is oval and is 3 cm in length

Glycerine agar —At the end of the first week the growth is 1 25 cm in diameter The central part is convoluted with white, chalky crust-like powder on the surface and around the central portion there is a halo of submerged growth

At the end of the second week, the growth is 2 0 cm in diameter including the submerged portion, but otherwise the character is unaltered

At the end of the third week the growth is 2 75 cm in length   It is more folded and shows many furrows distributed irregularly over the surface and the white powder appears to be thinner

At the end of the fourth week, the growth is 3 5 cm in length and the white powder is still further diminished (Plate XXII, fig 13)

At the end of the sixth week, the central part of one of the tubes shows a secondary faviform growth which is crinkled, glabrous and yellowish in colour

Both the tubes show slight salmon-pink coloration of the medium.

Liquid glucose- or maltose-peptone —The growth on liquid glucose-peptone (peptone 1 part, glucose 2 parts and water 100 parts) and liquid maltose-peptone (peptone 1 part, maltose 2 parts and water 100 parts) are of the same character as in other liquid media but the floating portion is whiter in glucose-peptone than in maltose-peptone or any other media, where it takes up a brownish colour   The inoculated masses within the liquid show the same puffy appearance in both of these, as in other liquid media

Anaerobic culture —On two occasions attempts have been made to grow the fungi in Sabouraud tubes , just after the inoculation the tubes were put in MacIntosh and Fildes' jar under complete anaerobic condition   Both the attempts were unsuccessful, as when they were withdrawn from the apparatus after 2 and 3 weeks respectively, they did not grow again in ordinary atmosphere   The control culture tubes gave a good growth

Partial anaerobic culture —Two Sabouraud test-tubes were inoculated with cultures of the fungus from Sabouraud slopes and partial anaerobic conditions were maintained

At the end of the first week, the growth was 0 75 cm in diameter disc-like with fine velvety down irregularly distributed on the surface, the furrows being very superficial

At the end of the second week, the growth was about 1 25 cm in diameter There were alternate lighter and deeper circular zones at the peripheral part and five or six very shallow, superficial radiating furrows (visible on transmitted light) on the surface

At the end of the third week, the growth was 1 5 cm in diameter with fine velvety chamois-leather surface

The second tube showed a growth 1 5 cm in diameter the centre covered with yellowish velvety down to an extent of 3 5 cm along its length and across the whole breadth of the slope   Furrows were practically absent but thickened areas could be seen by transmitted light   The nature of the growth was glabrous and covered with short velvety down

At the end of the sixth week, coarser powder had appeared in patches in a few areas on the velvety surface of the growth which was about 3 cm in length in the first tube and 5 cm in the second tube

Cultures of fungus on Sabouraud maltose-peptone agar with the oxygen supply limited by a rubber cap on the mouth of the test-tube —At the end of the first week, the growth was faviform, oval and 1 25 cm in breadth by 1·50 cm in length with fine white powder on the surface   There were about six shallow furrows radiating from the central point

At the end of the second week, the growth is 1 8 cm in breadth by 2 cm in length and is crossed by furrows from the central depression   The central portion was cerebriform and around it there was an irregular furrow which drains about 10 furrows distributed peripherally in a radiating manner

Fine fibrillar down had extended on the surface of and inside the medium beyond the central portion—to an extent of 0 5 cm on both sides   The growth was 3 cm in length along the slope

From the end of the third week onwards the character of the growth did not change much   The surface growth was about 4 cm in length with fine fibrillar down on the surface of the medium, becoming thinner towards the periphery

At the end of the sixth week, the powdery growth on the surface had extended and the whole growth with the peripheral fibrillar portion was about 5 cm in length

Culture on feather —Feather tubes were inoculated on the rachides of hen feathers with a culture taken from Sabouraud tubes They grew very slowly from the inoculated mass and the type of culture is that of an *Achorion*, though in one tube, very fine cobweb-like down was seen spreading along the length of the feather and its ridge

Animal inoculation —The healthy skin of a monkey, a rabbit, a mouse, guinea-pigs, and white rats were treated with an emulsion of culture from a Sabouraud tube, rubbed in with a blunt glass rod for five minutes over a clipped area

The monkey and the rabbit both showed lesions on the ninth day On the tenth day the lesions were definite though they were not prominent (photograph on eleventh day, Plate XX, fig 4) Later, ten to twelve scutula were definitely visible and each of them was roughly the size of a pea, but many of them were broken at the top

On the rabbit, scutula were removed by the animal continuously scratching itself and bleeding surfaces were left The lip of the rabbit and a part of the body other than the site of inoculation became infected by transmission from scratching the primary lesions on the back

The inoculation experiments were totally unsuccessful on the mouse and on two different white rats In the guinea-pigs there was only scab formation on the tenth day , these subsequently exfoliated, spontaneous cure resulting

Welled-slide culture —We studied the cultures of this fungus in three different media

1   Four per cent starch medium (viscid)
2   Semi-solid maltose-peptone (Sabouraud) medium
3   Synthetic medium without any amino acid (a modified formula of Czapek)

In all these micro-cultures there were two types of mycelia—one very thin without any end organs and the other thicker with characteristic end organs

In 4 per cent starch medium we had a satisfactory result as far as the growth of the fungus and its end organs was concerned The dichotomy was very characteristic on the meristematic part with formation of chandeliers such as is found in *Achorion schönleini*, and also intercalary and terminal oval or round chlamydospores were present But some of the terminal chlamydospores were of a peculiar funnel-shape, they were yellowish in some cases Arthrospores at close intervals and more commonly a few mycelial racquets and pectinate hyphæ were present (Plate XXIII, figs 14, 15, 16 and 17)

In synthetic media the mycelia were wavy in places they showed arthrospores in chains and intercalary chlamydospores in masses Terminal chlamydospores were also present though they were not very numerous

In Sabouraud's semi-solid medium with 0 2 per cent agar the cultures were very satisfactory showing all the end organs Here also some of the terminal chlamydospores were of the peculiar funnel-shape and some of them were yellowish as in the starch medium Mycelia showed aleurospores of a type of mycelial hernia but no free conidia There were also pectinate hyphæ and intercalary chlamydospores in chains Favic chandeliers in different stages of development were very common at the growing part (Plate XXIII, figs 14 to 17)

PLATE XIX

Fig 1

Fig 2

FIGS 1 and 2   Photographs of the patient
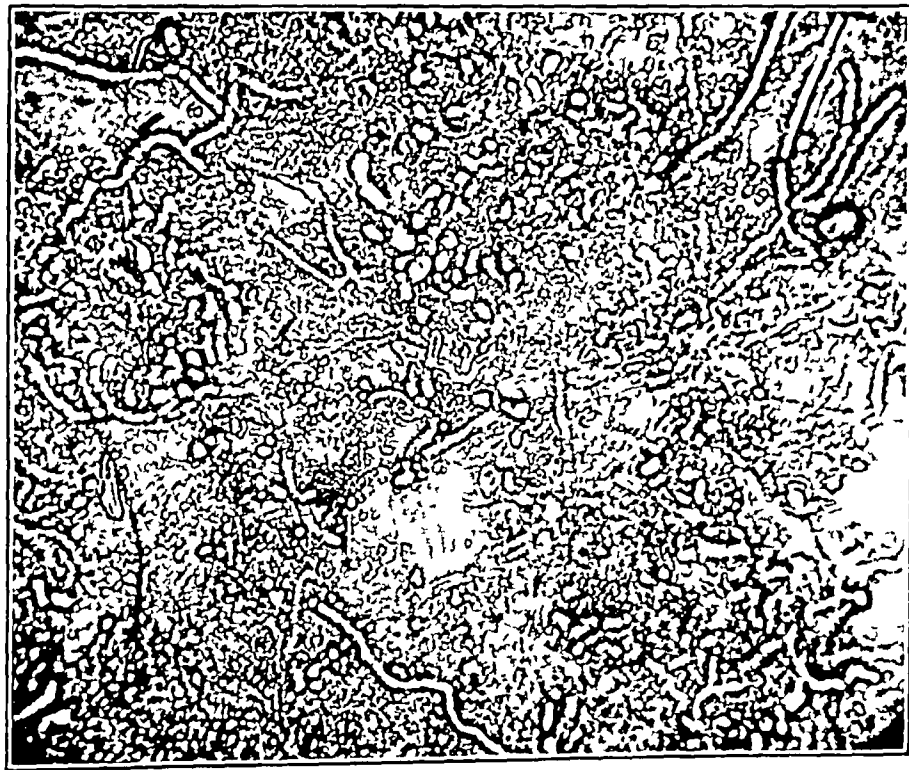
PLATE XX

Fig 4

Fig 3

FIG 3   Microphotograph of a scutulum cleared in sodium sulphide solution ($\times$ 400)

FIG 4   Photograph of monkey eleven days after inoculation

PLATE XXI

5   6   7

S   9   10
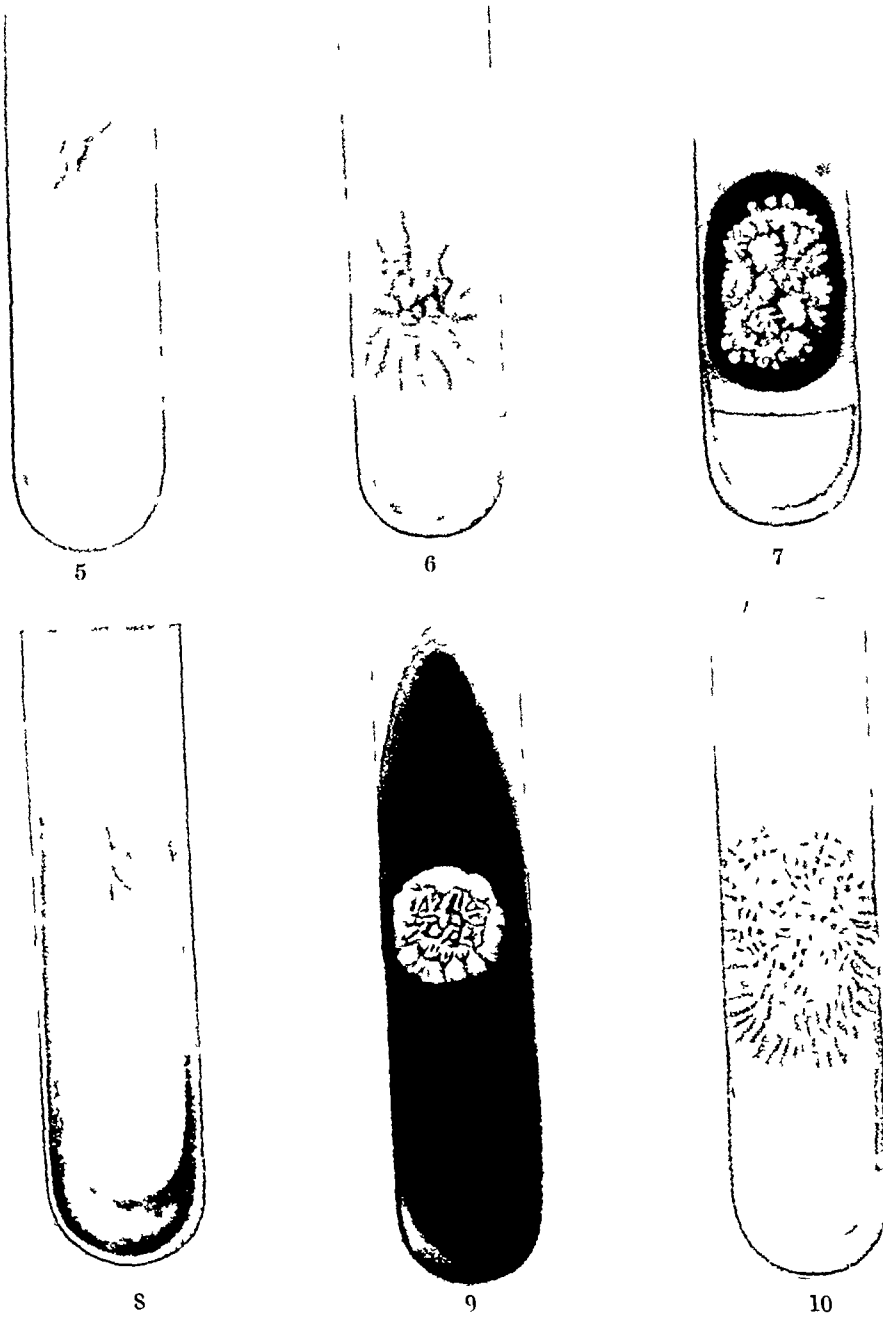
FIG   5   *A actoni n* sp on Sabouraud's maltose peptone agar, third week
       6   *A actoni n* sp on glucose agar third week
       7   *A actoni n* sp on inspissated serum, fourth week
       S   *A actoni n* sp on dh d agar, third week
   ,, 9   *A actoni n* sp on blood agar, third week
   ,, 10   *A actoni n* sp on Dorset's egg medium, fourth week

PLATE XXII
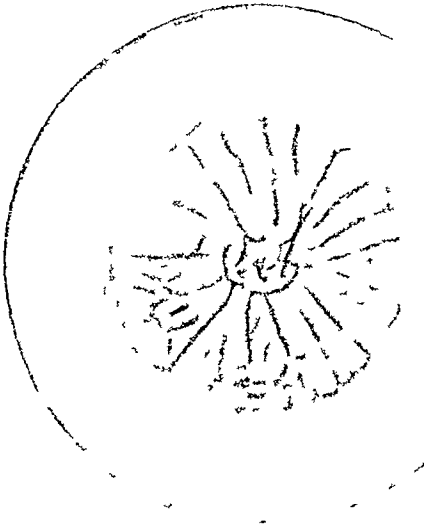
11
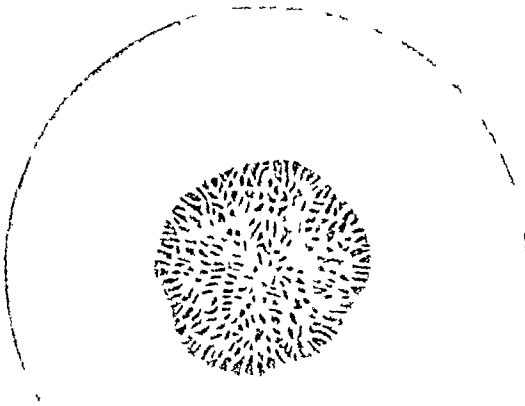
12
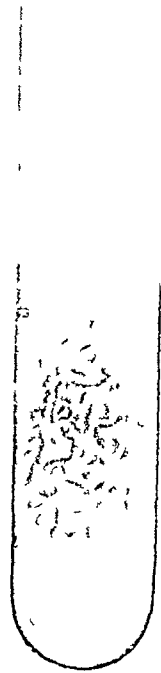
13

Fig 11   *A   actoni* n sp on a flask of Sabouraud's maltose-peptone agar
           fourth week
  „   12   *A   schonleini*
  „   13   *A   actoni* n sp on glycerine agar, fourth week

PLATE XXIII
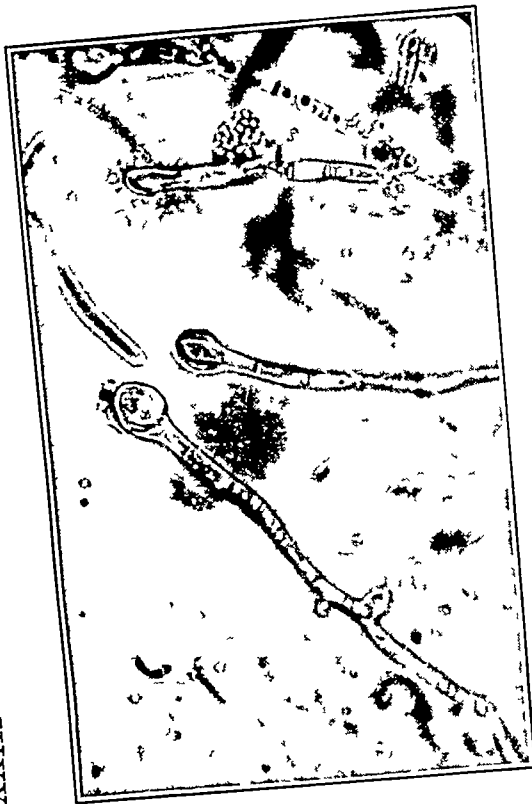
Fig 14

Fig 15

Fig 16
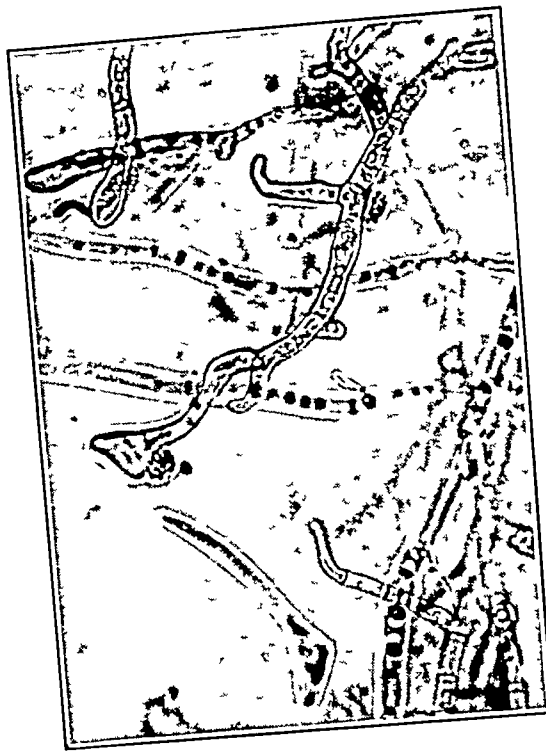
Fig 17

A actioni n sp photomicrographs of the end organs (× 400)

Fig 14   Developing chandeliers
Fig 15   Typical terminal chlamydospores
Figs 16 and 17   Special funnel shaped terminal chlamydospores

Preservation of the fungus —It has been shown that the fungus loses its characteristic chalky or fine powdery surface with age (4 or 5 months onward) and becomes glabrous and brown. The fungus maintains this glabrous appearance for a long time when not sub-cultured, and sub-cultures from the glabrous growth are themselves glabrous

The recovery of the fungus from this stage to the normal chalky or fine powdery growth can be brought about by breaking the culture into fine pieces and transferring it to a liquid synthetic medium *

Growth in this medium is granular at the bottom of the tube and after a month or more a portion transferred to a Sabouraud tube produces a typical growth
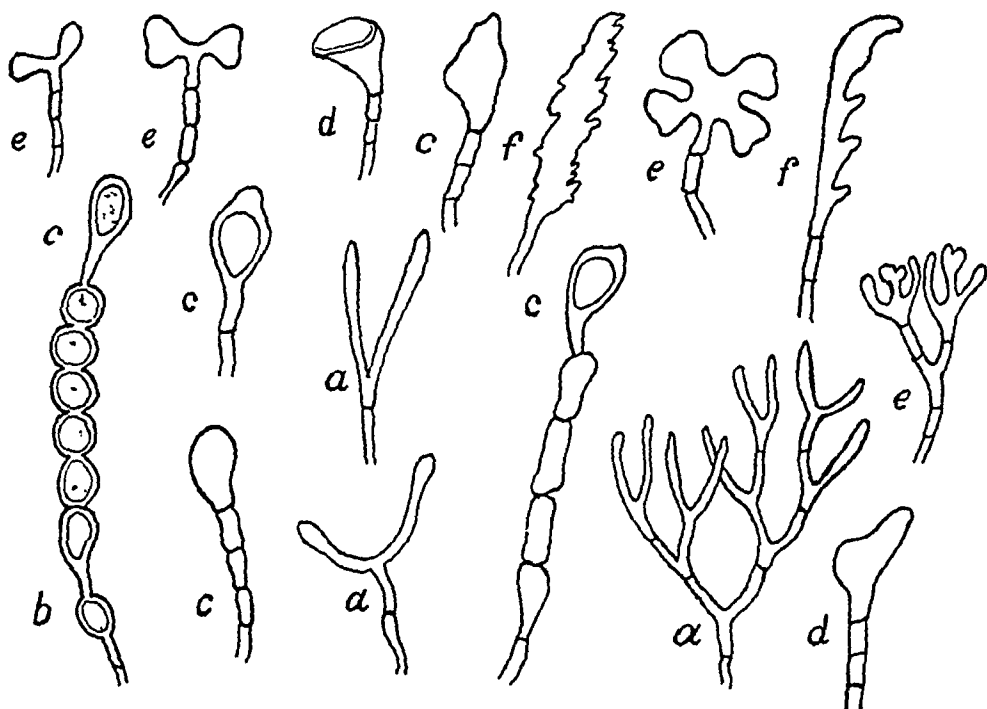
TEXT FIGURE —*A actom* n sp Drawings to show dichotomous branching and end organs

(*a*) Dichotomous branching (*b*) Intercalary chlamydospores in chains (*c*) Terminal chlamydospores (*d*) Special funnel shaped chlamydospores (*e*) Favic chandeliers in different stages of growth (*f*) Pectinate hyphæ

It will be seen from the study of the above culture reactions which have been summarized in the Table, that this species shows distinct differences from the hitherto recognized species of *Achorion*, it is accordingly proposed to name it *Achorion actom* n sp

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* (Modified Czapek's)

Water	1,000	c c
Sucrose	30	g
Sodium or potassium nitrate	2	g
Dipotass phosph	1	g
Potassium chloride	0 5	g
Magnesium sulphate	0 5	g

Favus in India.

TABLE.

Species	Diameter of spores	Colour of culture	Staining of media	Animal inoculation results	End organs (terminal chlamydospores)	In liquid media	Appearance when old
Our species	4μ to 6μ	Yellowish brown with white crusts	Brown when old	Monkeys and rabbits ++, guinea pigs ±, *mouse* −	Typical and also special funnel shaped type	Submerged portion flocculent	Glabrous
A formoseum	3μ to 4μ	First yellowish, then brown		Rabbits +, mice, guinea pig and monkeys −	Typical only		Downy
Grubyella (A) schönleini var *mongolica*		Maroon at first, later pale to typical yellow		Mice +++, guinea pig ±± and rabbits −	Typical only		Typical like *A schönleini*
A schönleini		Yellowish white	Does not stain	Dogs +++, rabbits +++, fowls +++, mice +++, guinea pig +	Typical only	Granular	Covered with white duvet*

Very susceptible +++
Moderately inoculable ++
Inoculable with difficulty +
Aborted lesions only ±
Negative −

* Our cultures of *A. schönleini* received from 'Centraal bureau voor schimmel cultures', Baarn (Holland), 4 years ago have not shown duvet, although they were kept for three months without sub culture

SUMMARY

1   A new species of *Achorion* has been isolated from a case of favus in India

2   In almost all media growths are faviform and covered with white chalky powder which is lost as the growth becomes older after which it is of the typical glabrous faviform type

3   The growths of our species in liquid media are different from that of *A schönleini,* the growth of the latter being granular

4   The end organs are like those of *A schönleini,* but there are in addition peculiar funnel-shaped terminal chlamydospores

5   The fungus is inoculable to the monkey and rabbit, and a slight superficial desquamation results on the guinea-pig   Mice and rats do not take the inoculation

6   We propose to name this species *Achorion actoni* n sp.

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STUDIES ON TYPHUS IN THE SIMLA HILLS

Part I.

INTRODUCTION

BY

LIEUT -COLONEL G COVELL, M D , D P H , I M S

(Inquiry under the Indian Research Fund Association)

THE TYPHUS GROUP OF FEVERS

NUMEROUS researches carried out during the last 20 years have shown that typhus fever in one or more of its forms is widely distributed throughout the world New facts regarding the epidemiology of the disease are continually being brought to light, and it is felt that a brief summary of the present position may be of value to workers in India

The so-called classical, historic or epidemic type of typhus has generally been classed apart from all the others on account of the fact that it is carried directly from man to man by the louse and occurs in epidemic form under circumstances of privation, over-crowding and generally insanitary conditions Recent researches have emphasized the essential similarity between the pathology of the classical type and that of the various non-epidemic forms of the disease Distinctions based on the degree of severity of the clinical symptoms have generally been abandoned, since it has been found that this may vary in the same type and in the same country at different periods

Much confusion has been caused on account of the many different names which have been given to the various members of the typhus group Megaw (1921, 1924 1925) has maintained that they should be classified by the name of the arthropod vector—louse-typhus, tick-typhus, mite-typhus, flea-typhus—but if this be done such names should be reserved for those types in which the vector has been definitely proved, otherwise the confusion is likely to be increased A disadvantage of this method of classification is that certain types of typhus have been proved to be carried by more than one vector , and future research may show that this is true of other members of the group

In all the members of the group the sera of the patients have been shown to agglutinate one or other of the strains of *B proteus* X   In the following table, taken from Felix (1935), a classification of the different members of the typhus group is given, based on the antigenic type of the virus, which constitutes a convenient summary of the position as far as is known at the present time —

Sub group	Type X19	Type XK	Type undetermined
Name of disease	Classical Epidemic Typhus Tabardillo, endemic typhus (Brill's) of U S A and Australia, Greece, Syria, Manchuria, Malaya (shop typhus), and Toulon (fiévre nautique)	Japanese River Fever (Tsutsugamushi fever of Japan, Malaya and Dutch East Indies), Malay scrub typhus, scrub-typhus of East Indies	Rocky Mountain Spotted Fever Sao Paulo endemic typhus*, fiévre bouton neuse, febbre erritiva, tick bite fever of S Africa, epidemic and endemic typhus of S Africa, Indian tick-typhus
Vector	Lice and rat fleas	Mites	Ticks, lice and rat fleas
Reservoir of virus	Rats Man	Field mice and rats	Rodents Dogs ? Ticks Man
Agglutination	X19 +++ X2 + XK —	X19 — X2 — XK +++	X19 + X2 + XK +

 * Sera from cases of Sao Paulo typhus has been shown to agglutinate X19 and XL (Lima) in high dilution, so that it is hardly correct to class it under the heading of 'Type undetermined' (Vide *Trop Dis Bull*, **32,** p 556)

 In the majority of cases the animal reservoir has been shown to be a rodent of some kind, and it has been suggested that the different properties of the viruses of the various types of typhus have been evolved from a single stock, by passage through different insects and hosts

 Zinsser (1935) has drawn attention to the remarkable variety of complex parasitic cycles by which the various members of the typhus group are communicated to man   Tsutsugamushi disease is conveyed to man by the bite of the mite, from field mice and rats, and kept alive in endemic regions from field mice to field mice by mites   Rocky Mountain spotted fever is conveyed to man by ticks, and is hereditarily transmitted by both male and female parents to their larvæ   Hence no animal reservoir is necessary, although, since various animals are known to be susceptible, one may exist   The fever of Sao Paulo, transmitted by ticks in Brazil, is probably identical with Rocky Mountain spotted fever Fiévre boutonneuse, which occurs in the neighbourhood of Marseilles and also in Roumania, is transmitted by ticks, and in this case also the virus passes from one

generation to the next without the need of an animal reservoir, though here again the dog as well as the rat may act in this capacity Finally, in what is known as ' true typhus ' two distinct types exist, the one carried from man to man by the louse, the other (sometimes called the murine type) carried from rat to rat by the rat louse and rat flea, by the rat flea on occasion to man, and from man to man by the louse The aetiological problem of typhus has been further complicated by the demonstration of inapparent infections in rats, dogs and man

In most members of the group the presence of *Rickettsiœ* has been demonstrated The researches of Zinsser and his collaborators have shown that *Rickettsiœ* are not highly specialized to any form of insect, but can survive for considerable periods in insects far removed from one another in zoological relationship

The results of research in Malaya during the last 10 years are of particular interest to workers in India Fletcher and Lesslar (1925, 1926) showed that there were two serological types of endemic typhus in that country, the one (urban or shop typhus) agglutinating *Proteus* X19 strains and not *Proteus* XK, whilst the other (rural or scrub typhus) agglutinates XK strains but not X19 The ' K ' or Kingsbury strain of *Proteus* had been brought out to the Malay States as an ordinary *Proteus* X19 culture in 1923 from the Bland-Sutton Institute, having been obtained from the National Collection of Type Cultures in 1921 When used by Fletcher and Lesslar, it was found to have changed its character, no longer generating indol in peptone water as do the majority of *Proteus* strains

Although the two types of typhus occurring in Malaya are thus serologically distinct, Anigstein (1933) has described an experiment in which a strain of the XK form derived from the blood of a human case apparently became transformed into the X19 form during a series of passages through rats and guinea-pigs Again, Lewthwaite (1932) has established a strain derived from wild rats, which when inoculated into rabbits produces agglutinins sometimes of the XK form and sometimes of the X19 form

Lewthwaite and Savoor (1934) have shown that in clinical, serological and epidemiological features tsutsugamushi disease in Malaya and rural or scrub typhus are closely similar, and conclude that ' the viruses of the two diseases are identical one with another, and with the *Rickettsia orientalis* described by Nagayo and his co-workers as the causal organism of the tsutsugamushi disease of Japan '

TYPHUS FEVER IN INDIA

Husband and McWatters (1908) published an account of outbreaks of typhus fever in Peshawar The disease broke out in 1905 in the First Mule Côrps, which had just returned from Tibet, and in 1906 there was a further epidemic affecting the First and later the Sixth Mule Côrps, about 120 cases being recorded in that year These authors also mention further isolated cases in the following winter, in men who had returned from leave from various places in the neighbourhood They refer to the occurrence of the disease in Kashmir in a valley at an elevation of 8,000 feet above sea-level, where it was well known among the natives The same authors note that epidemics which were probably typhus were described more than 50 years previously in the Yusufzai country and in Kohat, that the disease was identified by Fairweather in Rawalpindi Jail, and had often been recognized in Peshawar Jail At the Indian Medical Congress of 1894 it was stated that typhus

was endemic in the trans-Indus districts from Baluchistan to Yusufzai and Hazara, and in the Himalayan Hill Tracts, more especially Kulu (Hendley, 1894, Pisani, 1894)

All the epidemics referred to above occurred in the winter, from January to April, usually in the last three of these months   In the Peshawar epidemic the disease spread from one troop to another almost as readily as within the same troop Among the contacts many cases occurred, there being several instances in which all the contacts, 4 or 5 in number, were attacked   A detailed account is given of the clinical symptoms, and whatever doubts may arise regarding the records of earlier epidemics, there can be none with respect to the character of the disease here described as typhus fever

Hepper (1908) recorded 6 cases in Peshawar Jail in March of that year   The outbreak ceased on the adoption of drastic methods of disinfection, including the subjection of the iron cots to intense heat, and the burning of straw on the floors of the rooms, the object being to destroy bed-bugs, which were suspected to be vectors of the disease

Bradley and Smith (1912) described a case of typhus-like fever in a European soldier stationed at Jaffarpur camp, near Calcutta   Apart from this, there appears to be no further reference to the disease in India until McKechnie (1913) made his report on a series of 26 cases occurring in Bhim Tal and Sat Tal in the Kumaon Hills, chiefly among the servants of European visitors

In 1917 Megaw published an account of his own case, which he described as resembling Brill's disease   He contracted his illness in the same district where McKechnie's cases had occurred, and had been bitten by a tick 21 days before the attack   He suggested that the tick was probably *Rhipicephalus sanguineus* or *Hyalomma aegypticum*, and also that the fever described by McKechnie should be classed with Rocky Mountain spotted fever and Brill's disease as a sub-group of typhus fever

Phipson (1923) gave a detailed account of an outbreak of typhus in Simla, occurring between 10th November and 16th February   It comprised 16 cases, restricted to a single family, with a case mortality of 37 per cent   It was thought that the virus had been introduced by a healthy third party from a focus of infection 25 miles away, probably by means of infected lice   The sera of 12 of the cases were tested by the Weil-Felix reaction against *B proteus* X19, and all gave some degree of agglutination, the highest titre recorded being over 1   2,000   The sera of 100 healthy persons tested as controls gave no trace of agglutination in a dilution of 1   16, in any case

During the winter of 1923-24, Major F W Cragg, I M S , investigated an epidemic of typhus, believed to be louse-borne, in Kashmir   He fell ill with symptoms of typhus on his way back to Lahore, where he died on 23rd April, 1924

The various papers published by Megaw led to the recognition that fevers of the typhus group occur in widely separated parts of India, and cases have been reported from Calcutta, Angul (Orissa), Dacca, Darjeeling, Kalimpong, Akyab, Thayetmyo, Rangoon, Hyderabad (Deccan), Nagpur, Jubbulpore, Saugor, Balaghat, Pachmarhi, -Kamptee, Mut-Kuh and Padriganj (Central Provinces), Allahabad, Hamirpur, Cawnpore, Dehra Dun, Rutlam, Bellary, Trimulgherry, Secunderabad,

Trichmopoly, Bangalore, Poona, Ahmednagar, Madras Hill Tracts, Delhi, Murree, Malakand, Gilgit, Hyderabad (Sind), Kasauli, Sabathu and Dagshai (Simla Hills)

During the last few years a number of cases have been recorded among the troops in India, and Boyd (1935) has analysed a series of 110 cases which occurred in the Army in 1934   Serologically these were grouped as 43 XK cases, 14 X2 cases, and 51 X19 cases   Boyd concludes from the absence of co-agglutinins that the XK group is distinct , that the X2 group, in which co agglutinins regularly occur, probably does not have X2 as its main antigen, but as a group reaction to some other virus , and that the same applies to the Ahmednagar-Poona type of X19, whilst the Bangalore type of X19 is probably distinct   He thinks it possible that the X2 and X19 cases may have one unknown main antigen in common, and that the varying X2 and X19 titres are either individual idiosyncrasies in a group reaction, or are related to the passage of the virus through different vectors

TYPHUS FEVER IN THE SIMLA HILLS

The winter outbreak of typhus fever in Simla amongst a verminous family of Mohammedan vegetable sellers described by Phipson (1933) has already been discussed   MacNamara (1935) has given an account of 15 cases which occurred among British troops in Kasauli and the neighbouring cantonments of Sabathu and Dagshai in 1934   This was the third successive year in which a series of cases of typhus had occurred among the troops in this area   In 1932 there were 5 cases with 2 deaths at Sabathu   In 1933, there were 10 serologically proved and 4 clinically positive cases distributed among the three cantonments

In 1934, the first case commenced on 27th August, and the last on 1st October, the same seasonal period as in the two previous years   All the cases gave positive results with OXK by the Weil-Felix test, in titres varying from 1   150 to 1   25,000   All gave negative results with OX19   In no case was any infestation of the person or clothing of the patient by any insect noticed   In 12 of the cases there was no history of any bite   One case is noted as being ' badly bitten on the legs ', and another had been bitten by some insect behind the right ankle 14 days before admission to hospital   A third case, which the writer had the opportunity of examining closely, had developed a patch of dermatitis in the right lumbar region a few days before his attack   This was intensely irritating, but not painful (as is erroneously stated by MacNamara)

There is but little evidence as regards the incidence of the disease among the local population   It is known that fevers are prevalent during the month of September, but it has been assumed that these were of the typhoid group   One case occurred in an employee of the Central Research Institute in September 1934, which gave a positive Weil-Felix reaction for OXK in a dilution of 1   2,500, and it is probable that other cases formerly diagnosed as typhoid fever were really of the typhus group *

Two other cases, one a sub-assistant surgeon, the other a clerk, occurred during the winter of 1934-35   These both gave high titre agglutination with OX19, but were negative with OXK   The former case had returned from leave in the plains

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* At the present time (September 1935) there are two Indian patients in the Cantonment Hospital, Kasauli, suffering from typhus (XK type)

17 days before the onset of fever, but the incubation period of the typhus group is usually not more than 12 days, and it is probable that in both cases the disease was contracted in Kasauli

Thus it appears that in the Simla Hills, as in Malaya, there exist two types of typhus fever, the serum of one agglutinating strains of *Proteus* XK and not X19, whilst that of the other gives the opposite reaction   But, whereas in Malaya both types occur all the year round, in the Simla Hills each has a very definite seasonal incidence, the XK type occurring during the two months immediately following the rainy season, and the X19 cases occurring in mid-winter and early spring   Both types usually occur in sporadic form, but the winter type may cause group outbreaks or epidemics as in the case of that described by Phipson (*loc cit*)

As to the vector or vectors, in the majority of cases there is usually no definite history of an insect bite   The XK type of the disease occurs at a time when mosquitoes sand-flies ticks and fleas abound and are biting freely, so that in any case the history of an insect bite would not be remarkable   There is annually a great increase in the number of fleas just after the end of the rains   A strain of typhus has been isolated from rat fleas during the present investigation, but much more experimental work is necessary before it can be stated with certainty that the flea is the usual vector of the disease

With regard to the winter cases, the evidence suggests that the virus is transmitted by an insect which normally seldom infests man, such as the rat flea   But under conditions of extreme insanitation and poverty, as in the case of the Simla outbreak referred to above, transmission from man to man may be carried out via the louse, as in eastern Europe   This would explain the outbreaks recorded in the jails of northern India   and the epidemic described by Husband and McWatters in which the virus behaved like a contagious disease

SUMMARY

(1) A brief account is given of the ætiology of the typhus group of fevers, and the history of typhus fever in India is reviewed

(2) In the Simla Hills there exist two types of typhus, the one (XK type) occurring during the two months immediately following the rains, the other (X19 type) occurring in mid-winter and early spring   The latter type has been known to occur in epidemic form   The epidemiological evidence as to the insect vector is briefly discussed

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STUDIES ON TYPHUS IN THE SIMLA HILLS.

Part II.

THE WEIL-FELIX REACTION IN WILD RATS

BY

LIEUT.-COLONEL G COVELL M D, D P H, I M S

(*Inquiry under the Indian Research Fund Association*)

DURING the period 27th May to 15th August, 1935, the sera of 524 wild rats (*Rattus rattus* group) were examined by the Weil-Felix test   The rats were trapped at Kasauli, chiefly in the bazaar (246), Pasteur Institute Estate, 500 feet below Kasauli (79), Garkhal village, 3 miles from Kasauli (35), and Sabathu, about 8 miles from Kasauli (164)   Trapping at Sabathu was not commenced until 12th June

For the Weil-Felix reaction, concentrated alcoholized suspensions of *Proteus* X strains, obtained from the Enteric Laboratory, Kasauli, were used   All sera were tested against suspensions of OX19 and OXK, but only 122 against OX2 The Dreyer's tubes were kept in a water-bath at 50°C to 52°C for 4 hours, and then in an incubator at 37°C for 18 hours, being returned to the water-bath for a few minutes before the results were read, as recommended by Bridges (1935)   The lowest dilution of serum put up was 1   25   The results of the test for OX19 and OXK are summarized in Table I —

TABLE I

Titre	OX19		OXK	
	Number	Percentage	Number	Percentage
No trace, 1 25	416	79 4	1	0 2
15–22	29	5 5	3	0 6
25–45	33	6 3	55	10 5
50–110	30	5 7	397	75 8
125–225	10	1 9	57	10 7
250 and over	6	1 1	11	2 1
TOTALS	524		524	

In about 80 per cent of the cases there was no trace of a reaction with OX19 in the 1 25 tube, whereas it was usual to obtain standard or total agglutination in the 1 50 tube with OXK   It is probable that agglutination in a dilution of 1 50 with OX19 and 1 125 with OXK constitutes evidence of infection with a typhus strain in rats, but it was considered preferable to adopt a standard of 1 125 and 1 250 respectively, which yielded a positive result in 4 2 per cent of the cases Anigstein (1933),¹ who adopted a standard of 1 125 for both OX19 and OXK, obtained positive results in Malaya with this standard in 10 7 per cent out of 130 wild rats trapped in an endemic rural typhus area

As regards the 122 sera tested against OX2, 66 4 per cent gave no trace of agglutination in a dilution of 1 25, whilst a further 26 per cent yielded a titre of less than 50   In no case was the titre of OX2 the highest of the 3 strains tested

The titres of the 22 sera which gave a reaction of 125 and over with OX19, or 250 and over with OXK, are given in Table II   The results suggest that infection with both X19 and XK strains of typhus existed among the wild rats in this area, throughout the period of observation   Yet only XK cases have been recorded among human beings in the period August to October, and only X19 in winter Anigstein (1933) obtained similar results, about half of his positive cases among rats being of each strain, although only XK cases occurred among human beings in the area where they were trapped   Possibly the character of the strain may be modified by passage through the arthropod vector

<div align="center">TABLE II</div>

Date	Number of rat	Locality	Wtii Felix reaction		
			OX19	OXK	OX2
2nd June	32	Pasteur Estate	125	85	Not tested
5th ,,	47	Kasauli	350	85	Not tested
6th ,,	55	Kasauli	125	125	Not tested
8th ,,	68	Garkhal	250*	200	Not tested
21st ,,	128	Pasteur Estate	800	2,000	200
23rd ,,	145	Kasauli	0	250	0
2nd July	199	Sabathu	125	85	17
4th ,,	220	Sabathu	125	70	20
11th ,,	271	Sabathu	1,000	1 750	1,000
11th ,,	272	Sabathu	1,000	1,500	1,000
11th ,,	273	Sabathu	900	700	500

* This serum was not put up beyond a dilution of 1 250

TABLE II—*concld*

Date	Number of rat	Locality	WEIL FELIX REACTION		
			OX19	OXK	OX2
16th July	329	Sabathu	125	70	Not tested
16th ,,	333	Sabathu	175	35	0
17th ,,	337	Pasteur Estate	55	300	275
19th ,	364	Pasteur Estate	0	350	0
21st ,,	391	Sabathu	50	1,000	250
23rd ,,	399	Sabathu	50	250	Not tested
25th ,,	423	Sabathu	125	70	Not tested
25th ,,	427	Kasauli	175	800	500
2nd August	501	Kasauli	125	125	Not tested
6th ,, .	515	Pasteur Estate	0	250	Not tested
12th ,,	566	Kasauli	125	1,000	500

According to the standard adopted in Table II, the index of rats showing infection on the Pasteur Institute Estate was 6 3 per cent, at Sabathu 6 1 per cent, at Garkhal 3 per cent, and at Kasauli 2 4 per cent

It was noted that the 7 sera which yielded the highest titre all showed a considerable degree of hæmolysis The converse did not hold true, there being a number of sera which showed hæmolysis, but gave no more than the usual reaction

The testicles of 200 rats were examined macroscopically, but in no instance was there any evidence of a scrotal reaction comparable with that produced by the experimental infection of guinea-pigs In one case only there appeared to be some injection of the tunica vaginalis, with a slight increase in the amount of exudate This is contrary to the experience of Anigstein (1933), who noted enlargement and injection of the testicles with hæmorrhagic exudate into the tunica and occasional hæmorrhages into the polar fat in 9 out of 200 rats trapped in an endemic rural typhus area in Malaya

The sera of 11 mice (*Mus musculus*) and of 14 squirrels (*Sciurus palmarum*) gave Weil-Felix reactions similar to that usually obtained with wild rats, i e , no agglutination with OX19 in a dilution of 1 25, but usually standard or total agglutination with OX19 in the 1 50 tube None exceeded this titre Normal guinea-pigs and white rats gave reactions of a similar order

Normal rabbits, however, showed a marked difference Out of 53 tested, only one showed any trace of agglutination with OX19 , whilst with OXK, 36 were completely negative, and of the remainder none showed any trace of agglutination beyond a dilution of 1 25

SUMMARY

(1) The results of the Weil-Felix tests on 524 wild rats trapped in Kasauli and the neighbourhood are detailed   The usual result was nil in a dilution of 1   25 with *Proteus* OX19, and standard or total agglutination in the 1   50 tube with OXK

(2) In 22 cases there was agglutination in a dilution of 1   125 or over with OX19, or 1 ، 250 or over with OXK   This is considered to indicate evidence of infection with a typhus strain   By this standard the index of rats showing infection at the Pasteur Institute Estate was 6 3 per cent, at Sabathu 6 1 per cent, at Garkhal 3 per cent, and at Kasauli 2 1 per cent

(3) Results of the Weil-Felix test in the case of a number of mice, squirrels, white rats, guinea-pigs and rabbits are also given

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STUDIES ON TYPHUS IN THE SIMLA HILLS.

Part III

A STRAIN OF TYPHUS RECOVERED FROM WILD RATS

BY

LIEUT -COLONEL G COVELL, M D , D P H , I M S

(*Inquiry under the Indian Research Fund Association*)

THE virus of typhus fever has been demonstrated in the brains of wild rats in Mexico, the United States, the Mediterranean basin (Toulon, Piraeus, Alexandria, Syria), Manchuria, Malaya and South Africa

In the present investigation attempts were made to establish a strain (1) by inoculating emulsions of rats' brains into the anterior chamber of the eye of rabbits, by the method of Nagayo and his collaborators (1931) and (2) by inoculating emulsions of rats' brains intraperitoneally into guinea-pigs Five attempts to produce a specific ocular reaction in rabbits, two animals being used on each occasion, failed although in two rabbits a positive Weil-Felix reaction resulted Out of nine attempts to infect guinea-pigs intraperitoneally, two to four animals being used on each occasion, one experiment resulted in a single guinea-pig developing a moderate rise of temperature from the 13th to 15th days, accompanied by a slight scrotal reaction, whilst another resulted in the establishment of a strain in guinea-pigs, as recorded below

Workers in Malaya had experienced great difficulty in establishing strains of typhus in laboratory animals until Lewthwaite (1932) used guinea-pigs fed on a vitamin-deficient diet, as recommended by Zinsser, Castaneda and Seastone (1931) It was feared that a similar difficulty might be experienced here and the guinea-pigs were therefore placed on a diet of bran and gram only, but this regime had only been instituted on the day the animals in which the strain was initiated were inoculated so that it could have had little or no effect on the course of the first passage After the first few passages, a normal diet was given and infection was secured without difficulty

On 24th July, 1935, four guinea-pigs were inoculated intraperitoneally with a pooled emulsion in normal saline of the brains of 3 wild rats, which had been trapped at Sabathu   The sera of the rats gave the following Weil-Felix reaction —

	OX19	OXK
Rat 307	100	45
Rat 399	50	250
Rat 402	100	70

Guinea-pigs I and II developed fever on the 7th day, followed by a typical scrotal reaction on the 8th   Guinea-pig III, which had lost over 25 per cent of its weight since the date of inoculation, died on the 8th day, whilst guinea-pig IV, which had lost 20 per cent of its weight, showed a febrile reaction on the 7th day, and died suddenly on the 8th day whilst its temperature was being recorded

The two surviving animals were killed for passage on the 11th and 13th days after inoculation respectively   The strain, which is now in its 10th passage, has been maintained in guinea-pigs chiefly by the inoculation of spleen emulsion or testicular washings intraperitoneally, as these appear to be more virulent than brain emulsion or blood

REACTION IN THE GUINEA-PIG

During the course of the investigation, 76 guinea-pigs have been inoculated with the rat strain virus   Of these, 15 showed no symptoms, 48 reacted with scrotal inflammation and fever, 10 with fever only, whilst 3 died between the 6th and 8th days with no symptoms except loss of weight   The incubation period was usually from 5 to 8 days   the shortest period observed being 4 days and the longest 12 days   The length of the incubation period varied considerably according to the material inoculated   The average periods were   testicular washings 4 9 days   spleen emulsion 5 8 days, brain emulsion 9 3 days   Blood was used as the inoculum in 4 cases   but none of the animals reacted   All the inoculations into guinea-pigs were given intraperitoneally

The fever generally began abruptly, the temperature rising to about 103°F within 24 hours, but it was exceptional for it to exceed 104°F at any time   During the first 4 passages it was unusual for the pyrexia to persist for more than 3 or 4 days, but in the later passages it has continued for 10 to 12 days on several occasions

Loss of weight was by no means a constant feature, though frequently about 20 grammes was lost during the febrile period, this being usually regained by the end of the second week after inoculation   On 3 occasions there was progressive loss of weight, without fever or scrotal reaction, commencing on the third or fourth day and terminating in death on the sixth to eighth day, by which time the animal had lost 25 to 30 per cent of its weight   Post-mortem examination of one of these animals showed hæmorrhages in the lungs, injection of the tunica vaginalis and enlargement of the testicles   Passage of the brain into another guinea-pig resulted

PLATE XXIV
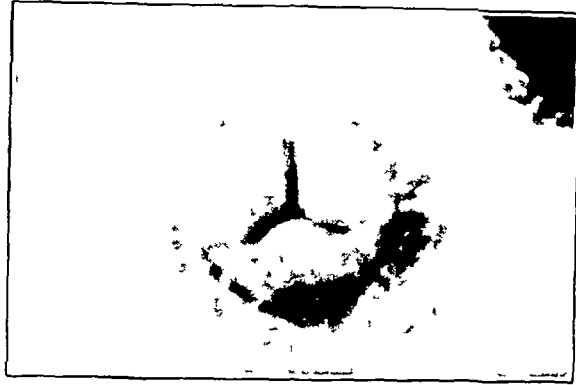
FIG 1   Genitalia of normal male guinea pig

FIG 2   Scrotal reaction in guinea pig   Same
scale as above

in fever and typical scrotal reaction on the ninth day, and numerous *Rickettsiæ* were seen in scrapings from the tunica vaginalis

The typical scrotal reaction was observed in 63 per cent of the cases, but there were marked variations in its frequency in different passages Thus, out of 41 animals used in the 3rd, 5th, 6th and 7th passages, 37 (90 per cent) showed this reaction, whilst out of 10 animals used in the 4th and 8th passages only 3 reacted in this manner The onset was generally sudden, usually coinciding with the rise in temperature, though it sometimes preceded or followed this within a few hours It was a common experience to observe a slight swelling of the scrotum, perhaps on one side only, in the evening, and to find the whole organ greatly swollen and inflamed on the following morning (*see* Plate XXIV) At the height of the reaction the skin over the scrotum was usually dusky red and shiny, and sometimes the œdema involved the prepuce also Occasionally there were petechial spots on the surface, and on one occasion there was a purple hæmorrhagic patch involving the whole of one side of the scrotum The swelling and inflammation usually subsided as abruptly as it had begun, and there was often no outward sign of it by the fourth day after the first symptoms had been noticed

On incising the skin, the tissues were found to be œdematous and adherent, rendering dissection somewhat difficult On opening the tunica, the parietal layer was always found to be much thickened and inflamed, and frequently showed hæmorrhages Sometimes it was difficult to strip the parietal from the visceral layer, and if the process had lasted 3 or 4 days the surfaces not infrequently presented a 'bread and butter' appearance when pulled apart The testicles were injected and enlarged, and there were often hæmorrhages into the polar fat There was also an increase in the exudate which was usually hæmorrhagic and sometimes gelatinous If the post-mortem was delayed until some days after the symptoms had subsided, there was generally but little sign of anything abnormal, except for some thickening of the parietal tunica

In the majority of cases hæmorrhagic changes were noted in the lungs, ranging from minute petechiæ to hæmorrhagic infarction The spleen was generally congested, with prominent tubercles, whilst in a number of cases a greyish film of exudate was noted on the surface Congestion of the meninges of the brain was a constant feature

Smears were prepared from scrapings made with a knife from the tunica vaginalis of guinea-pigs which showed a scrotal reaction, but considerable difficulty was at first experienced in demonstrating the presence of *Rickettsiæ* satisfactorily In preparations made at the height of the scrotal reaction the whole field was frequently obscured by the presence of enormous numbers of granules, staining red with Giemsa, which Mooser (1928) has shown to be derived from destroyed endothelial leucocytes It was not until preparations were made from the tunica vaginalis of white rats, killed arbitrarily on various days from the 5th to the 13th after inoculation, that a satisfactory demonstration of intracellular organisms was obtained Subsequently, equally good results were secured from guinea-pigs killed immediately the scrotal reaction was noticed The best results were obtained with unfixed smears, thoroughly dried and stained with Giemsa for 2½ to 3 hours

The organisms seen showed a very close resemblance to those described as *Rickettsiæ* by other workers and a comparison of the smears with preparations

made from the tunica of rats infected with the murine type of Mexican typhus, kindly sent by Dr H Mooser, left no doubt as to their character The following forms were seen —

    (1) Minute bacillary forms This was the type most frequently seen within the endothelial cells They were usually in diplo-bacillary form, and stained blue or light purple with Giemsa Sometimes they were joined at an obtuse angle, and occasionally they were arranged end to end in chains On two occasions a filamentous type, presumably formed in this way, was seen within an endothelial cell

    (2) Somewhat thicker and longer forms with rounded ends, showing distinct bipolar staining

    (3) Slender rods of varying length, containing metachromatic granules

As a rule, where numbers of intracellular organisms were present, it was excep-tional to find extracellular forms, except where these were in a group obviously spilled from a ruptured cell Conversely, where extracellular forms were scattered throughout the smear, it was rare to find intracellular forms also In some preparations certain cells were crammed with organisms to such an extent as to obscure the nucleus of the cell

REACTION IN THE WHITE RAT

During the investigation 29 white rats were inoculated intraperitoneally with passage virus from guinea-pigs, either spleen emulsion or testicular washings being used Of these, 8 were killed for experimental purposes on or before the 10th day Of the remainder 10, or nearly 50 per cent, died between the 11th and 16th days The brain of one of those which died on the 12th day was passaged into a guinea-pig, which developed typical scrotal reaction and fever on the 5th day The sera of three which were found in a dying condition between the 13th and 16th days gave a positive Weil-Felix reaction for OX19 in dilutions of 1 500 (two) and 1 350 (one) It seems probable that most, if not all, of the deaths were due to infection with typhus virus

In only one case was any rise of temperature noted This animal had fever from the 8th to 12th days after inoculation, and died on the 16th day It is evident that pyrexia is rare in white rats infected with this strain of typhus

In no case was any sign of a scrotal reaction observed, although typical intra-cellular *Rickettsiæ* were found in scrapings from the tunica vaginalis of animals killed from the 5th to 13th days after inoculation

The sera of 19 rats tested for the Weil-Felix reaction between the 9th and 21st days showed agglutination for OX19 in 13 cases The highest titres observed were 1 500 (twice, on the 15th day) and 1 1,000 (once on the 16th day) None showed any significant agglutination for OXK Four sera tested before the 9th day gave negative results with both OX19 and OXK

REACTION IN THE RABBIT

The pooled brain emulsion from the 3 wild rats from which the strain was initiated was inoculated into the anterior chamber of the eye of two rabbits Neither developed any sign of a specific ocular reaction The sera of the two

TABLE 1.

Results of Weil-Felix tests on rabbits

NUMBER OF RABBIT.

Days after inoculation	A 5 OX19	A 5 OXK	B 5 OX19	B 5 OXK	C OX19	C OXK	D 1 OX19	D 1 OXK	D 2 OX19	D 2 OXK	D 4 OX19	D 4 OXK	D 5 OX19	D 5 OXK	D 6 OX19	D 6 OXK	D 7 OX19	D 7 OXK	D 8 OX19	D 8 OXK
0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	250	0	0	0	50	0	125	0	0	0	0	0	0	22	0	0	85	30	25	22
15	125	0	50	0	250	0	125	0	25	0	0	0	50	0	0	0	1,000	0	500	0
20	125	0	25	15	125	0	50	0	25	0	0	0	30	25	0	17	250	0	250	25
25	50	0	0	0	50	0	50	0	15	0	0	0	25	22	25	0			125	17
30	25	0			25	0	25	0	0	0	0	0	17	15	17	0	75	0	30	25

animals before inoculation was completely negative to both OX19 and OXK (1 25 dilution), but one of them gave a reaction with OXK from 1 70 to 1 50 between the 14th and 24th days Eight rabbits were inoculated intra-ocularly with testicular washings from first passage guinea-pigs, but none developed the specific ocular reaction, nor did any of them show a positive Weil-Felix reaction

Ten rabbits were inoculated intraperitoneally with emulsions of spleen only, or of a mixed emulsion of spleen brain and testicles of passage guinea-pigs The results of the Weil-Felix tests on these animals are given in Table I.

As has been found by other observers, none of the rabbits showed any symptoms as the result of the inoculations although the dose was a large one Each of the last 5 animals received an emulsion containing the whole spleen of a passage guinea-pig and the remainder an approximately equivalent amount of a pooled emulsion of different organs

REACTION IN THE MONKEY

Two monkeys [*Silenus (Macacus) rhesus*] were inoculated subcutaneously with a mixed emulsion of brain, spleen and testicle from a passage guinea-pig One received 10 c c and the other 5 c c of the emulsion The former showed a rise of temperature to 103 2°F on the 8th day, 103 6°F on the 9th, and 104 0°F on the 10th, after which it dropped to normal (i e , 101 5°F to 102 5°F) This monkey gave a Weil-Felix reaction for OX19 of 1 55 on the 10th day, 1 200 on the 15th and 1 175 on the 20th The other animal showed no pyrexia, but its serum agglutinated OX19 in a dilution of 1 50 on the 10th day (*see* Table II)

TABLE II

Results of Weil-Felix tests in monkeys

Days after inoculation	MONKEY No I		MONKEY No II	
	OX19	OXK	OX19	OXK
0	0	0	25	35
10	50	25	55	25
15	35	25	200	25
20	25	25	175	17
25	17	25	85	30
30	17	25	60	35
35	0	17	50	25
40	0	20	50	25

Neither animal showed any sign of illness   Blood was taken from the monkey which had fever on the 10th day after inoculation, and 2 c c was inoculated intra-peritoneally into each of two guinea-pigs   One of these reacted with scrotal reaction and fever on the 14th day   The spleen of this animal was passaged into another guinea-pig, which developed typical scrotal reaction and fever on the 9th day, after which the strain was dropped   A rabbit inoculated with a mixed emulsion of spleen and brain from the first guinea-pig gave a Weil-Felix reaction for OX19 of 1 125 on the 15th day

SEROLOGICAL REACTIONS OF THE STRAIN

The Weil-Felix reactions given by the 3 wild rats from which the strain was initiated suggest that two of them were infected with an X19 type and the third with XK   One of two rabbits which were inoculated intra-ocularly with the pooled emulsion of the brains of the 3 rats gave the Weil-Felix reactions shown in Table III —

TABLE III

Results of Weil-Felix tests in rabbit 399R

Days after inoculation	WEIL-FELIX REACTION	
	OX19	OXK
0	0	0
9	0	0
14	0	70
19	0	60
24	0	50
29	0	15

This was the only animal which gave a positive reaction with OXK during the investigation

On the other hand, positive reactions with OX19 have been given by 13 white rats, 7 rabbits and 2 monkeys   In the case of the rabbits and monkeys, where repeated tests were carried out on each animal, the typical waxing and waning curve of the Weil-Felix reaction, regarded as diagnostic of typhus infection by Fletcher and Lesslar (1926), was noted   The highest titres recorded were 1 1,000 (once in a rabbit, once in a white rat), and 1   500 (once in a rabbit, twice in white rats)

It seems probable that the strain was originally a mixture of two types, one producing agglutinins for X19, the other for XK, but that the XK type died out after the first passage

The sera of 21 passage animals were tested against X2, but none gave a significant reaction

SUMMARY

(1) A strain of typhus has been recovered from the brains of wild rats trapped in an endemic typhus focus, and it is now in its 10th passage in guinea-pigs

(2) Typical scrotal reaction and fever has been observed in 63 per cent of the guinea-pigs inoculated with the virus

(3) Intracellular organisms exhibiting the characters described as typical of *Rickettsiœ* by other workers on typhus have been found in scrapings from the tunica vaginalis of guinea-pigs and white rats infected with the virus

(4) The reactions produced in rabbits, white rats and monkeys are described The white rat is particularly suitable for demonstrating *Rickettsiœ* and for the Weil-Felix test

(5) The serological reactions of the strain are described

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SPONTANEOUS TUBERCULOSIS IN LABORATORY MONKEYS

BY

K V KRISHNAN, M B , B S , M R C P , D B , D SC ,

Professor of Malariology and Rural Hygiene, All-India Institute of Hygiene and Public Health, Calcutta

A FATAL outbreak of spontaneous tuberculosis occurred among the monkeys of the Malaria Department of the All-India Institute of Hygiene and Public Health, Calcutta, during the year 1934-35   In this outbreak 14 out of 15 monkeys died after an average contact of 35 weeks with tubercular guinea-pigs which were being kept in the same room   The circumstances leading to the outbreak being interesting it was considered worth while publishing a detailed account of them, together with the post-mortem and other findings made in the course of the investigation

During the years 1932 to 1934 the experimental monkeys belonging to the writer were housed in the *ground floor* animal-room of the Institute   In those years his monkeys shared the animal-room with the mice and hamsters of the Kala-azar Department   Although in all over forty-four monkeys were kept in the room for periods varying from 3 to 12 months and at death of the animals post-mortem regularly performed, only one animal was detected to be suffering from tuberculosis   The history of this monkey was as follows   Soon after its arrival it was used in a malaria experiment   A month after its recovery from the disease, splenectomy was done   It had a severe relapse of malaria, developed hæmoglobinuria and died   Post-mortem showed scattered, multiple caseating tubercles in the lungs and tracheo-bronchial glands   It was a *Silcnus nus* monkey that had recently been imported from Singapore and inquiries suggested that it might have contracted the tubercular infection either during the voyage or prior to it   As none of the other animals obtained from the same dealer showed any evidence of tubercular infection the presumption was that the infection was not contracted by the animal while under the care of the animal-dealer   In April 1934, the writer began to house his monkeys in the animal-room on the *first floor* of the Institute   At the time one-half of this room was being occupied by guinea-pigs belonging to the Tuberculosis Inquiry   The writer felt that it was unsafe to keep his monkeys with tubercular guinea-pigs because of the ready susceptibility of the former to the disease   But, as accommodation was limited and as his monkeys

could not be housed elsewhere, they were kept along with the tubercular animals The monkeys were kept singly in iron cages and these were arranged in rows one foot apart on the floor in one-half of the room In the other half the guinea-pigs were kept in small non cages on shelves These shelves were kept three feet or more away from the monkey-cages A sketch of the animal-room and the arrangement of the cages is given in Plate XXV Two animal attendants looked after the animals, the monkeys were cared for by one and the guinea-pigs by the other Thus there was no chance of infection being conveyed to the monkeys through careless handling or mixing up of food by the attendants But on the other hand there was every likelihood of contamination of the food occurring by contact with the floor of the room The monkeys invariably upset the trays containing their food and later picked up the food from the floor The earlier infections at least were probably contracted in this way Out of a total of 21 monkeys brought into the room from time to time in batches (9 in April, 4 in August, 4 in December and 4 in May) and belonging to three species (*S rhesus, S irus* and *S radiatus*) six died within 4 to 8 weeks of arrival of induced malarial hæmoglobinuria, and post-mortem examination of these showed no evidence of tuberculosis Of the remaining 15, 14 contracted tuberculosis and died The first died at the end of four months (in September 1934) and second two months later (in November 1934) After this deaths occurred at varying intervals till all the animals except one had died of the disease The monkey that escaped infection was a *Silenus radiatus* The following is a brief history of the tubercular monkeys and their post-mortem findings —

1   *Silenus radiatus* (Rd₁) was the first monkey to die of tuberculosis It was brought into the animal room on 12th April, 1934, received cholesterol and olive oil by mouth for six weeks and then used in malaria experiments It suffered from *Plasmodium knowlesi* infection off and on for a period of 12 weeks and then recovered completely from it On 29th July, 1934, it was splenectomized but no relapse of malaria occurred Although it was looking perfectly normal previously it was found dead on 18th October, 1934, and post mortem revealed evidence of widespread tuberculosis On opening the chest a large caseating mass involving the ribs, sternum and skin was found The pus from it contained acid fast bacilli Both lungs were studded with small caseating tubercles and the tracheo bronchial glands were also infected In the abdomen the mesenteric glands were found caseating and acid fast bacilli were found in their smears The liver, spleen and kidneys showed no macroscopic lesions The intestine was congested and near the ileo cæcal junction the mucous surface was congested and superficial ulcers present Sections of these showed acid fast bacilli

2   *Silenus rhesus* (R₂) was the second monkey to die of tuberculosis It was brought into the animal-room on 6th August, 1934, and received an injection of blood containing *P knowlesi* It had several attacks of malaria followed by a long period of latency On 16th October, 1934, its spleen was removed and a mild relapse of malaria ensued, which was cured after treatment A few weeks later the animal developed a severe diarrhœa Examination of the stools revealed the presence of intestinal flagellates in large numbers (*Trichomonas* and *Giardia*) On 13th December, 1934, it died and the post mortem showed the following lesions In the abdomen—caseating mesenteric glands were present The liver showed yellowish caseating areas of size of a pea in large numbers The large intestine was highly congested and superficial ulcers of the mucous membrane were also noticed Sections and smears of infected organs showed acid fast bacilli In the thorax there were no caseating glands In the lower lobe of the right and the middle lobe of the left lung few tubercles were seen Smears from these contained few acid fast bacilli

3   *Silenus irus* (Ir₁) was the third monkey to die of tuberculosis It came into the animal room on 12th April, 1934, and received cholesterol and olive oil for two months It was then infected with *P knowlesi* and had a severe attack of malaria It was cured of it after treatment On 6th September, 1934, it was splenectomized to induce relapse, but it did not develop any It was apparently in normal health but on 12th January, 1935, it was found dead Post mortem showed the following lesions In the abdomen—the liver, kidney, mesenteric glands and the intestines all showed distinct caseating tubercles In the chest—one lung (the right) showed a cluster of small tubercles in the middle lobe surrounded by a pneumonic patch Fluid was present in the pleural

cavity   The corresponding tracheo bronchial glands did not show any macroscopic evidence of tubercular infection   Smears of the glands showed no acid fast bacilli

4   *Silenus rhesus* (R_9) was the fourth monkey to die of tuberculosis   It came into the animal-room on 6th August 1934, but was not used in any experiment   It began to lose weight from 16th December, 1934, and died on 17th January, 1935   Post mortem showed a large mass of caseating glands behind the sternum in which acid fast bacilli were present   Both lungs showed caseating tubercles   In the abdomen there was no macroscopic lesion suggesting a tubercular infection in any of the organs

5   *Silenus radiatus* (Rd_3) was the fifth monkey to die of tuberculosis   It was purchased in 1933 and was used in malaria experiments   It had a high degree of acquired immunity   It was splenectomized on 20th April, 1933, but did not relapse   Injection of *P knowlesi* infected blood resulted in a transient infection which was spontaneously overcome   It was taken over by the writer for obtaining blood for experimental purposes   It came from the ground floor to the first floor animal-room on 12th April 1934, and between that date and its death on 17th May, 1935, it had a number of cardiac punctures done to it   It was quite robust in health when it died and the following lesions were discovered on post mortem   The pericardium was distended with fluid of straw colour which on injection into a guinea pig failed to show tubercle bacilli   The tracheo bronchial gland was found enlarged and caseating and smears from it showed tubercle bacilli   A single hard tubercle of the size of a split pea was found in the middle lobe of the right lung and it was surrounded by a small red area of consolidation   Other than this no other lesion was seen in any of the other organs in the chest or abdomen

6   *Silenus rhesus* (R_9) was the sixth monkey to die of tuberculosis   It was brought into the animal room on 10th December, 1934 and was given an injection of *P knowlesi*   On 22nd January, 1935 it was splenectomized and an intense infection of *P knowlesi* resulted   It was treated and after a number of relapses it finally got rid of the infection   On 21st April, 1935, it was noticed that the animal was getting thin, and on 5th July, 1935, it died   Post mortem showed the following lesions   In the abdomen—there was a large mass of mesenteric glands of the size of a closed fist   The cheesy pus from the glands showed acid fast bacilli   The liver was riddled with caseating tubercles of the size of a split pea   Both kidneys showed numerous caseating foci   The mesenteric glands were all caseating   The pancreas showed a caseating mass of the size of a marble   The intestine near the cæcum showed ulceration of the mucous surface   In the chest—the glands on the back of the sternum were enlarged and caseating   Bases of both lungs showed clusters of small tubercles   The pleural cavity contained a few c c of straw coloured, blood tinged fluid in which acid fast bacilli were present

7   *Silenus rhesus* (R_6) was the seventh monkey to die of tuberculosis   It was admitted into the animal room on 6th August, 1934, and was used in malaria experiments   It had several severe attacks of *P knowlesi* infection from which it finally recovered after prolonged treatment   On 25th January 1935, it was splenectomized and it had a severe relapse of malaria , from this also it recovered   About two months later it began to get thin and died on 10th July, 1935   Post mortem showed the following lesions   In the abdomen—the mesenteric glands were enlarged and caseating   The intestine was congested in places and showed typical tubercular ulcers from which acid fast bacilli were recovered   The liver and the other abdominal organs showed no macroscopic lesions   In the chest—both lungs were studded all over with tubercles   There was a big cluster of tubercles in the lower lobes of both lungs   Tubercles were more numerous in the right than in the left lung   The pericardium was filled with straw coloured fluid and was adherent in some places to the lung   Caseating foci were seen in the pericardial sac

8   *Silenus irus* (I_2) was the eighth monkey detected to be suffering from tuberculosis   It was brought into the animal room on 12th April 1934, but was not used in any experiment   While it was apparently in good health, on 21st July, 1935, it was splenectomized but the spleen was found riddled with tubercles   It was therefore killed on 24th July, 1935, and the post-mortem showed the following lesions   In the abdomen—the liver showed four caseating areas of the size of a split pea   The kidney was normal looking but the mesenteric glands were caseating   In the chest—the right lung alone showed a group of tiny tubercles surrounded by a red area of consolidation

9   *Silenus rhesus* (R_{10}) was the ninth monkey to die of tuberculosis   It was brought into the animal room on 10th December, 1934, and splenectomized on 22nd January, 1935   It had its blood repeatedly taken before and after splenectomy for cholesterol estimation   On 18th July, 1935, it was injected with blood from another monkey showing *P knowlesi* and on 29th July, 1935, it developed a heavy infection and died of hæmoglobinuria the following day   The post-mortem showed the following changes   In the abdomen—several mesenteric glands were found enlarged and caseous   The liver was studded with numerous caseating tubercles   The kidneys showed a few pin point caseating areas   The intestines were normal   In the chest—the right lung was completely studded with small miliary tubercles but the left did not show even one suspicious area   The tracheo bronchial glands were enlarged and caseous   Acid fast bacilli were found in the smears of all infected tissues

10　*Silenus rhesus* (R$_{13}$) was the tenth monkey to die of tuberculosis　It was brought into the animal-room on 21st May, 1935　On 23rd July, 1935, it was splenectomized and one week later given an injection of *P knowlesi*　On 6th August, 1935, it died of malarial haemoglobinuria　Post mortem showed in the chest two caseous areas of the size of a split pea each surrounded by a red area of consolidation of the size of a rupee in the right lung　Smears from these showed acid fast bacilli　The tracheo bronchial gland on that side was slightly enlarged and caseous　There was no other evidence of tuberculosis in any of the other organs

11　*Silenus rhesus* (R$_{11}$) was the eleventh monkey to die of tuberculosis　It was brought into the animal room on 10th December, 1934, and was given cholesterol in olive oil for two months　During this period its blood was repeatedly taken for estimation of cholesterol　After a time the animal was noticed to be getting thin and developing a cough　On 29th July, 1935, it was given an injection of blood containing *P knowlesi* and it died of malarial haemoglobinuria on 7th August, 1935　Post mortem showed extensive tubercular lesions of the miliary type in almost all the organs　In the abdomen fluid was present in the peritoneal cavity, mesenteric glands were caseous, the liver was studded with small caseating tubercles, both kidneys showed many pin point caseous areas and the spleen had also a few tubercles　In the thorax fluid was found both in the pleural and pericardial sacs which on injection into a guinea pig caused the death of animal from tuberculosis　Both lungs were adherent to the chest wall in several places and showed extensive miliary lesions　The tracheo bronchial glands were enlarged and caseous and the glands behind the sternum were also caseous　The pericardial sac was adherent to the lung and showed one tubercle

12　*Silenus rhesus* (R$_{12}$) was the twelfth monkey to die of tuberculosis　It was brought into the animal room on 10th December, 1934, and was given cholesterol in olive oil for two months like No 11　Some time after completion of the course the animal was found to be getting thin and to have cough and dyspnoea　So it was not used in any experiment　It died on 11th August, 1935, and the post mortem lesions were very similar to those of No 11　In the abdomen the liver, spleen and kidneys were all riddled with numerous miliary tubercles　The mesenteric glands were caseating and the intestines showed ulceration　There was fluid in the abdominal and pleural cavities and in the pericardial sac　Both lungs showed numerous miliary tubercles　The lungs were adherent in some places to the chest wall　The tracheo bronchial glands and the posterior mediastinal glands were caseating　The larynx showed tubercles　The diaphragm and the pericardium also showed lesions in one or two places

13　*Silenus rhesus* (R$_1$) was one of the oldest surviving monkeys in the laboratory　It came into the animal room on 12th April, 1934, and was given cholesterol in olive oil for 4 weeks　It showed a high blood cholesterol and was immediately used in a malaria experiment　It had an attack of severe malaria from which it recovered completely after treatment　A period of latency ensued and during this period it was resistant to super infection　On 11th January, 1935, it was splenectomized and as a result developed a relapse for which it was treated　It recovered completely and all attempts to re infect it failed　Although it was in perfect health it was killed on 2nd September, 1935, as it was necessary to write this report　On post mortem examination the only lesion detected was a completely calcified area of the size of a pea in the upper part of the middle lobe of the right lung, the other organs were normal　In the pericardium there was about 10 c c of clear straw coloured fluid which on injection into a guinea pig showed tubercle bacilli to have been present

14　*Silenus rhesus* (R$_8$) came into the animal room on 6th August, 1934, and was used in malaria experiments　It had several attacks of malaria off and on till 20th December, 1934, and then it recovered completely and was resistant to reinfection　On 18th January, 1935, it was splenectomized and a severe relapse followed　It was treated and cured of this condition　All attempts to re infect it failed　On 12th August, 1935, when it was apparently in normal health the animal coughed and brought out a blood clot　Examination of the clot showed no tubercle bacilli　On 2nd September, 1935, it was killed and post mortem showed evidence of fibroid tuberculosis　In the chest the tracheo bronchial glands were enlarged and hard with yellowish areas　There was a hard fibro caseous mass of the size of a rupee in the upper lobe of the right lung which was adherent to the chest wall and on separation showed a cavity　In the left lung there were a few hard small tubercles　In the peri cardial sac there was clear straw coloured fluid　In the abdomen the liver and kidneys both showed scattered hard tubercles　The mesenteric glands were enlarged hard and caseating

SYMPTOMATOLOGY AND PATHOLOGY OF TUBERCULOSIS IN MONKEYS

Tuberculosis in monkeys as observed in this outbreak ran a rapid course　The period of illness was short and most of the animals died within a period of 35 weeks　In most cases there were very few symptoms and no suspicion of tuberculosis was

PLATE XXV
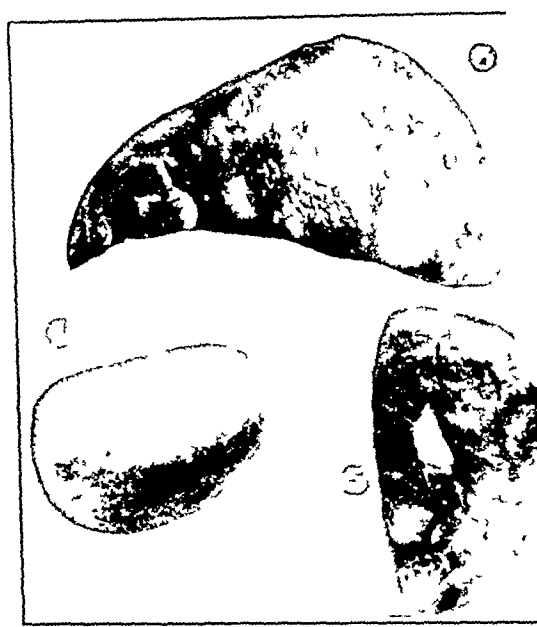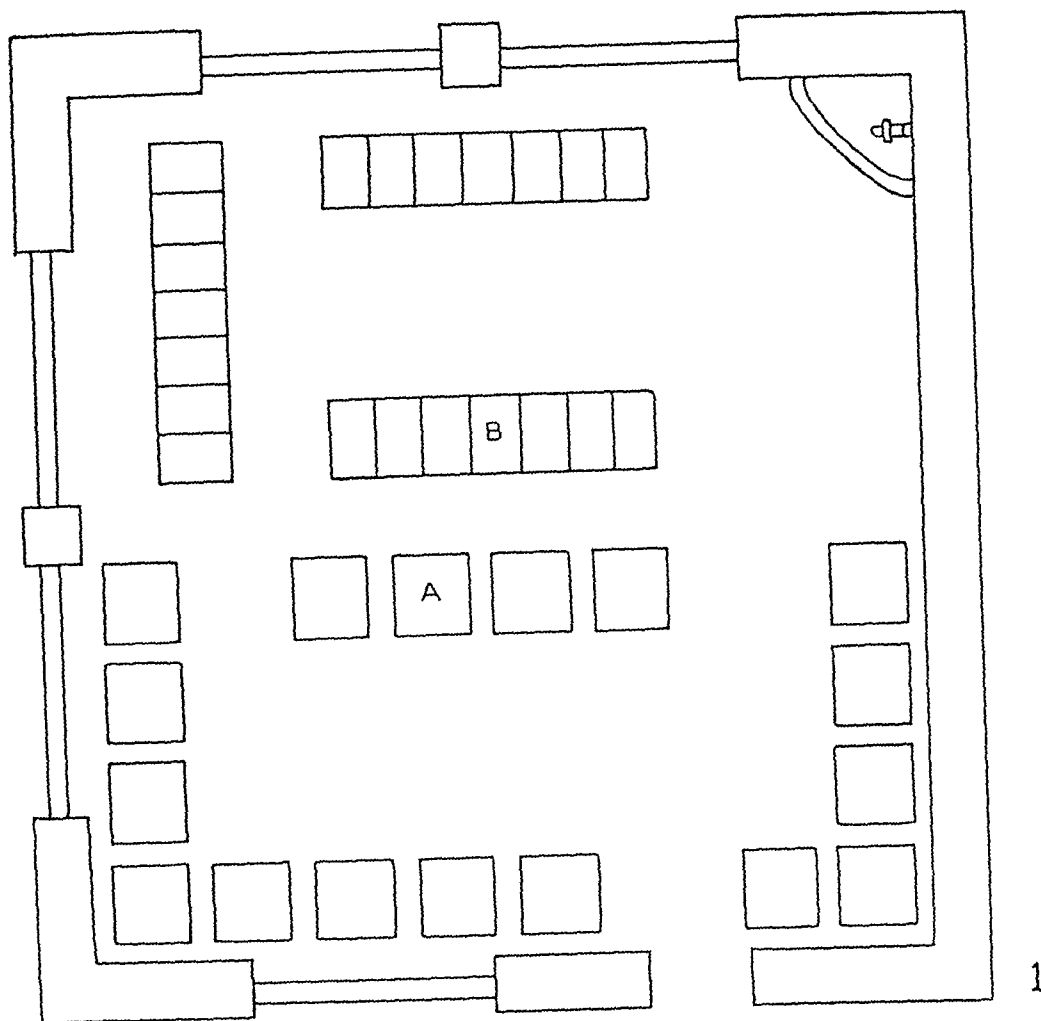
(1) The animal room on the first floor of the All India Institute of Hygiene and Public Health showing the arrangeme of monkey cages (A) and guinea pig cages (B) (2) Liver showing multiple caseating tubercles (3) Enlarged spleen showi multiple tiny tubercles in a monkey that died of malaria (4) A kidney showing tubercles (5) Spleen of a non mala monkey showing tubercles

PLATE XXVI

(1) Lung showing miliary tubercles   (2) Lung showing dorsal view showing glandular involvement   (3) Lung showing clusters of miliary tubercles and extensive pneumonic consolidation   The lung looked and felt like a liver   (4) Lung showing cavity   It was adherent to chest wall and on separation was found to be filled with pus   (5) A hard fibro caseous tubercle and caseating trachco bronchial gland   (6) A hard and calcified tubercle

aroused except at post-mortem   While 6 out of 14 showed symptoms, the other 8 looked normal until death   Emaciation was noticed in 6 cases, cough and dyspnœa in 2 cases (the last in the series), diarrhœa in 1 case (No 2) and spitting of blood in 1 case (No 14)   As regards the pathological lesions they resembled closely the lesions of acute tuberculosis of susceptible animals   They were mainly of the miliary type and dissemination was widespread   As a result of low resistance milia and nodule formation was rapid, caseation early and fibrosis and calcification infrequent   The gross macroscopical changes seen in the animals are given in the histories and some of the more important lesions have been photographed and presented in the Plates XXV and XXVI   The following is a brief tabular summary of the lesions —

Glandular infection was multiple extensive and common   Cervical glands were found caseating in 1 case, tracheo-bronchial glands in 9 cases and mesenteric glands in 10 cases   The serous membranes were fairly frequently attacked   The pleura was adherent to the lung or chest-wall or both in 6 cases and fluid was present in the pleural cavity in 3 cases   The peritoneum was affected in 2 cases and fluid present in it as well   The pericardium showed tubercles and adhesions in 3 cases and fluid was present in the sac in 2 cases   The lungs were affected in all cases Cavity was found in 1, healed fibrosed tubercle in 1   calcified tubercle in 1, miliary tubercle in 9   and massive tubercular pneumonic consolidation in 2   The diaphragm showed lesion in 1 case and also the larynx   As regards the abdominal organs superficial ulceration of the intestinal mucosa with presence of acid-fast bacilli was noticed in 7 cases, small caseating tubercles were found in the spleen in 2 out of 4 cases (most of the other monkeys being splenectomized), in the adrenal in 1 case in the pancreas in 1 case, in the kidneys in 6 cases and in the liver in 8 cases   In addition to the above in one case a large caseating mass involving the ribs, sternum and the skin of the chest was detected

DISCUSSION

Spontaneous tuberculosis in monkeys —In European countries spontaneous tuberculosis in monkeys kept in captivity for some time is of frequent occurrence The incidence of tuberculosis among the monkeys in the Zoological Gardens of London, Philadelphia and other western cities and in the various laboratories in which these animals are used for experimental purposes confirm this view (Scott, 1930)   In the Philadelphia Zoo Fox states that the average duration of life of monkeys was 11 months and practically every monkey died of the disease within a year if exposed to infection   Calmette in France, emphasizes the fact that in tuberculosis experiments on monkeys there is always the fallacy of spontaneous infection   This he thinks can be avoided by selecting tuberculin negative monkeys and by the use of recently imported animals from India   Indian monkeys in his experience are generally free from the disease , out of 52 freshly imported Indian monkeys that he tested tuberculosis was found in three only, whereas the incidence was much higher in monkeys which had been with the animal-dealer for some time after importation (Griffith, 1931)   In the London Zoological Gardens many years ago the incidence of tuberculosis in monkeys was quite high   About 30 per cent of the monkeys died of tuberculosis within a year and a very much higher percentage within 3 years   At present, however, due to greater care and attention of the

animals, the incidence has been reduced greatly In the zoological gardens and laboratories in India as far as the writer can gather tuberculosis in monkeys is not of common occurrence Inquiries made in the Calcutta Zoo where there are over 100 monkeys elicited the fact that death rate in monkeys from tuberculosis is less than 1 per cent per annum The same is also the experience of laboratories where monkeys are kept for experimental purposes In the writer's own experience although he had previously used over 100 monkeys on different occasions he met with only one case of tuberculosis and this too in an imported monkey from Singapore and not in an indigenous monkey Even in the present outbreak the first six animals belonging to the group that died of malaria a few weeks after their arrival in the laboratory showed no evidence of tuberculosis If these are taken as a representative sample of the monkeys at the time of purchase then it may be argued that probably none of the animals were infected at the time of their arrival in the laboratory and that all of them contracted the infection during their stay in the animal room in contact with the tubercular guinea-pigs This view is further supported by the fact that some other monkeys not belonging to the batch, but purchased from the same animal-dealer, and kept in contact with leishmania infected mice and hamsters in the ground floor animal-room, showed no evidence of the disease Therefore, this outbreak of spontaneous tuberculosis in monkeys kept in the first floor animal-room along with tubercular guinea-pigs is of special interest While there are several published records of spontaneous tuberculosis in monkeys from countries outside India (Kalbfleisch, 1929-34 , Nieberle, 1932 , Finkel Dey, 1931 , Griffith, 1931 , Scott, 1930), there are none as far as the writer is aware from this country

Source of infection —From the foregoing discussion it will be clear that the source of infection in the present outbreak was primarily the infected guinea-pigs It is possible that later in the outbreak the source might have been infected monkeys This shows that it is very undesirable to house experimental monkeys in the same room as tubercular animals If it is done outbreaks such as the present one may occur with disastrous consequences

Mode of infection —With regard to this there is mainly the post-mortem findings and the impression gained by the writer during the investigation While in some animals because of the nature and severity of the lesions in the abdominal organs and the relative scarcity of lesions in the lungs it is probable that the root of entry was intestinal, in others the evidence is definitely in favour of the respiratory route In 5 out of 14 monkeys (Nos 2, 3, 6, 8 and 9) the route was probably alimentary and in 9 out of 14 monkeys (Nos 1 4, 5, 7, 10, 11, 12, 13 and 14) the route was probably respiratory As the lesions in most animals were of the generalized miliary type, it is rather difficult to say by which route the organism first gained entry By whatever route it entered it soon got into the lymphatics and blood stream and finally got localized in the different organs In a few cases, however, there was distinct evidence that the route of entry was either respiratory or alimentary The impression gained was that in the earlier cases the route of entry was intestinal and in the later cases (because monkeys with symptoms came later in the series) the disease spread from monkey to monkey through droplet infection It was noticed that the monkeys kept nearest to the tubercular guinea-pigs and to other infected monkeys were more severely infected than those further removed from these sources of infection

The type of acid-fast bacillus —Cultures were made from the post-mortem material in six cases and the acid-fast bacilli present isolated   The strains were tested and typed (culturally and by animal passage) and found to be of the human type   As all the guinea-pigs in the room were infected with *M tuberculosis human* the finding of the same strain in all the monkeys lends further support to the view that their infection was probably contracted from the guinea-pigs

Susceptibility of different species of monkeys to tuberculosis —In the present outbreak 3 species of monkeys were involved and all appeared to be equally susceptible   Ten out of 10 *Silenus rhesus*, 2 out of 3 *Silenus radiatus* and 2 out of 2 *Silenus irus* contracted the disease   The type and distribution of lesions, in all species were the same   In only one   a *Silenus rhesus* was there a healed lesion which had gone on to calcification

The effect of splenectomy on the course of tuberculosis —It will be seen from a perusal of the histories of the monkeys that both splenectomized and non-splenecto-mized animals were present in the group and were equally attacked with tuber-culosis   Because the first few animals to die of tuberculosis were splenectomized animals it was thought that possibly the removal of the organ increased their susceptibility to the disease   But a careful study of the incidence and severity of the disease as well as the duration of life after contact of the animals in the two groups showed no marked difference between the two   As regards incidence 10 out of 10 splenectomized and 4 out of 5 non-splenectomized contracted the disease The average duration of life after starting exposure to tuberculous infection was 35 weeks in the splenectomized animals and 39 weeks in the non-splenectomized In the splenectomized group 60 per cent showed extensive lesions while in the non-splenectomized group 50 per cent showed correspondingly severe lesions   There was no difference in the distribution or type of lesions in the two groups   From this the writer presumes that so far as the effect of splenectomy on the incidence of tuberculosis goes, the course of infection is not materially altered by the removal of the organ

Since making these observations the writer has looked up the literature on the subject and he finds that his findings are in agreement with that of the majority Many of the reports are in German journals not available here   The two reports in English seen by the writer may be referred to however   Foot (1923) studied the effect of injecting tubercle bacilli in splenectomized and non-splenectomized rabbits and found that the course of infection did not vary materially in the two groups except that the animals in the former group tended to die off a little sooner than those in the control group   This is in agreement with the writer s experience On the other hand, Hayashi and Takeda (1933) found that after giving a sub-cutaneous injection of 100th of a mg of human tubercle bacillus to splenectomized and non-splenectomized guinea-pigs, 1 out of 6 of the latter and 3 out of 4 of the former showed lesions in the bone-marrow   From this the workers conclude that after splenectomy the lesions in the bone-marrow were more numerous   The writer's experience does not corroborate this   There are many more reports published on the subject and the following may be quoted for reference (Brandis, 1929 , Sarvan, 1931 , Roso, 1932   Fabris, 1934)

The effect of malaria on the course of tuberculosis —A few observers (Kyriasidis, 1931 , Collari, 1933) have noted that when chronic malarial patients contract tuberculosis the progress of the latter disease is slow and c the fibroid type , this

they presume is because the body of a malarial subject probably offers a powerful resistance to the development of tuberculosis   In the present series there were 4 normal and 10 malarial monkeys   A comparison of the tubercular lesions in the two groups showed no very marked difference   Most members of the two groups suffered from the same type of acute miliary tuberculosis   In 3 monkeys belonging to the malarial group however, there was some evidence of fibrosis or calcification These monkeys had previously suffered very severely from malaria but unlike the others had developed a very high degree of immunity to super-infection   In view of Scott's statement that in his experience fibroid phthisis and healed tubercles are absolutely unknown in monkeys the presence of such lesions in the 3 animals referred to above (R_1, R_8 and Rd_3) may be attributed to the influence of acquired immunity to malaria   If this view is correct then it may be stated that acquired resistance to malaria may, in some cases, be helpful in checking the progress of tuberculosis and reducing its severity   On the other hand, if the effect of acute tuberculosis on the course of malarial infection is considered, it is found that animals suffering from miliary tuberculosis do not show any resistance to malarial infection, but that in them malaria runs a very severe and fatal course   Jemma (1933), has made the observation that acute malaria aggravates the course of tuberculosis The observations recorded here do not contradict this but merely show that animals suffering from acute tuberculosis developing malaria die very rapidly   It is difficult to say whether death is due to the severity of the malarial infection or to the aggravation of the tubercular process   From a study of the course of acute malaria in monkeys suffering from chronic fibroid or healed tuberculosis, the writer has got the impression that resistance to tuberculosis is helpful in checking the severity of malaria and in enhancing acquired immunity to malaria   It is possible that the mechanism of immunity in the two diseases is closely allied and that further studies on the subject may be helpful in solving the question

RÔLE OF CHOLESTEROL IN TUBERCULOSIS

It will be evident from a perusal of the histories of the monkeys that the out-break of tuberculosis occurred while they were being used in an experiment on the rôle of cholesterol in malaria   On account of this intercurrent infection, the cholesterol results were vitiated and the experiment was given up   In a few cases, however, it was found that the cholesterol readings obtained brought out some points of importance relating to tuberculosis   one of these was the blood-cholesterol values in normal and tubercular monkeys and the other was the effect of cholesterol feeding on the course of acute miliary tuberculosis   With regard to the first it was found that while the cholesterol value in whole blood of monkeys varied from 140 mg to 170 mg per cent, the average value for monkeys suffering from severe widespread tuberculosis varied from 80 mg to 110 mg per cent   This result it may be observed is in general agreement with the findings for humans   In man blood-cholesterol is unaltered in the early mild cases but is very much lowered in the more severe and rapidly progressing cases   With regard to the second question it will be seen that 4 monkeys were fed on cholesterol and olive oil for periods varying from 1 to 2 months after the commencement of exposure to tubercular infection   Of these, two were subsequently used in malarial experiments and the other two were not   The latter showed the severest and most extensive lesions in the series   As regards the former, which were used in malarial experiments, one

showed a moderate infection and the other a healed lesion It is difficult to say whether this difference in the type of tuberculosis in the two groups is due to the attacks of malaria which the latter suffered from , if so resistance to malaria may be said to increase the resistance to tuberculosis But so far as the effect of cholesterol on the course of tuberculosis goes, the impression gained by the writer is that over-feeding with cholesterol and olive oil may help in intensifying the course of tubercular infection A reference to the published literature on the subject subsequent to gaining this impression showed that although a large number of people had used cholesterol in the treatment of tuberculosis the results obtained are by no means uniform The experimental studies on rabbits by Levinson (1931) show that over-feeding with cholesterol and olive oil does not favourably influence the course of tuberculosis nor does it protect the animals against the disease Luden (1917) came to the same conclusion He showed that a diet which reduces blood-cholesterol increases lymphatic resistance and that which increases blood-cholesterol weakens it Tunnicliff (1923) also found that intravenous injection of small doses of cholesterol had a stimulating effect on phagocytosis, while large doses had a depression influence Krishnan, Ghosh and Bose (1936) have shown that hypocholesterinæmia is more favourable to phagocytosis than hypercholesterinæmia From these it appears that if the reticulo-endothelial system and lymphocytic response play a part in checking the tubercular process then over-feeding with cholesterol and olive oil is likely to intensify the tubercular infection as observed by the writer

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AN INVESTIGATION OF CHEAP 'WELL-BALANCED' DIETS

BY

W R AYKROYD

AND

B G KRISHNAN

(*Nutrition Research Laboratories, I R F A , Coonoor, S India*)

DURING recent years, as a result of the economic crisis, considerable attention has been given, in a number of countries, to the problem of devising cheap ' well-balanced ' diets   Numerous pamphlets have been issued by public health authorities, charitable organizations, etc , which instruct the poor and unemployed how to purchase an adequate diet for a few shillings, dollars, kronen, etc , per week, and often provide a variety of specimen menus for daily consumption , in India a pamphlet on ' balanced diets ' has been issued by the Bombay Presidency Baby and Health Week Association, and achieved a wide circulation   As a rule ' well-balanced ' diets recommended as suitable for consumption are drawn up by consulting tables of food analyses given in standard textbooks, and applying generally accepted dietary standards   Due regard must, of course, be given to the food habits of the country concerned, for it is of little use to recommend diets which, however cheap and however rich in valuable food factors, are not in conformity with dietary custom   In India the problem of devising satisfactory cheap ' well-balanced ' diets is somewhat more complicated, first, because we lack at present precise knowledge of the composition of many Indian food-stuffs, particularly as regards mineral salts and vitamins, and, secondly, because it is doubtful whether dietary standards put forward by American and European physiologists are applicable *in toto* to India   The present paper records an attempt to devise and test in the laboratory a number of cheap ' well-balanced ' diets suitable for general consumption in India

TEST DIETS

The first step was to work out a number of food mixtures which, on the basis of figures given in McCarrison's (1931) book ' Food ' had an energy value of about 2,700 to 2,800 calories, approximating to the daily requirements of an average man   The test diets, 12 in number, are described in Table I   With the exception of diets 11 and 12, they resemble Indian diets in general composition, and are based

TABLE I.

Composition of test diets

Diet 1

Raw polished rice	12	oz
Cambu (*Pennisetum typhoideum*)	6	,
Dhal arhar (*Cajanus indicus*)	1	,
Coco nut oil	1	,
Jaggery (unrefined cane sugar)	1	,
Root vegetables	2	,
Leafy vegetables	8	,
Dried skim milk	0 5	,
Butter milk (sour skim milk)	10	,
Ground nut (*Archis hypogea*)	1	,
Soya bean	1	,
Sodium chloride	0 25	,

Diet 2

Raw polished rice	12	oz
Atta (whole wheat flour)	6	,
Dhal arhar	1	,
Coco nut oil	1	,
Jaggery	1	,
Root vegetables	2	,
Leafy vegetables	8	,
Dried skim milk	0 5	,
Butter milk	10	,
Ground nut	1	,
Soya bean	1	,
Sodium chloride	0 25	,

Diet 3

Raw polished rice	12	oz
Cholam (*Sorghum andropogon*)	6	,
Dhal arhar	1	,
Coco nut oil	1	,
Jaggery	1	,
Root vegetables	2	,
Leafy vegetables	8	,
Dried skim milk	0 5	,
Butter-milk	10	,
Ground nut	1	,
Soya bean	1	,
Sodium chloride	0 25	,

Diet 4

Raw polished rice	12	oz
Ragi (*Eleusine coracana*)	6	,
Dhal arhar	1	,
Coco nut oil	1	,
Jaggery	1	,
Root vegetables	2	,
Leafy vegetables	8	,
Dried skim milk	0 5	,
Butter milk	10	,
Ground nut	1	,
Soya bean	1	,
Sodium chloride	0 25	,

Diet 5

Cholam	18	oz
Soya bean	2	,
Dhal arhar	2	,
Jaggery	2	,
Leafy vegetables	8	,
Root vegetables	2	,
Coco nut oil	1 5	,
Butter milk	6	,

Diet 6

Ragi	16	oz
Soya bean	2	,
Dhal arhar	2	,
Jaggery	1	,
Leafy vegetables	8	,
Root vegetables	2	,
Coco nut oil	1 5	,
Butter milk	6	,

TABLE I —*concld.*

Diet 7

Cambu	18	oz
Soya bean	1	,,
Dhal arhar	0 5	,,
Jaggery	3	,,
Coco nut oil	1 25,	,,
Leafy vegetables	8	,,
Root vegetables	2	,,
Butter milk	6	,,

Diet 8.

Polished raw rice	15	oz
Soya bean	3	,,
Dhal arhar	3	,,
Jaggery	0 5	,,
Leafy vegetables	8	,,
Root vegetables	2	,,
Coco nut oil	1 5	,,
Butter milk	6	,,

Diet 9

Ragi	16	oz
Black gram (*Phaseolus mungo*)	6	,,
Jaggery	1	,,
Amaranthus gangeticus	2	,,
Spinach	2	,,
Lady's fingers (*Hibiscus escu-lentus*)	2	,,
Bitter gourd (*Momordica charantia*)	2	,,
Colocasia antiquorum	1	,,
Pumpkin	1	,,
Plantain fruit	2	,,
Gingelly oil (*Sesamum indicum*)	1 5	,,
Butter milk	6	,,

Diet 10

Atta (whole wheat flour)	18	oz
Lean meat	2	,,
Whole milk	20	,,
Gingelly oil (*Sesamum indicum*)	1	,,
Ghee	1 5	,,
Colocasia antiquorum	2 6	,,
Potatoes	2 7	,,
Carrots	2 7	,,
Cabbage	8	,,
Plantain fruit	4	,,
Dhal arhar	1	,,

Diet 11

Atta, chapatties smeared with butter, fresh raw cabbage, fresh raw carrots, sprouted Bengal gram (*Cicer arietinum*), fresh cow's milk 5 0 c c per rat daily and meat twice a week

Diet 12

Purified starch	60 parts	
Casein	20 ,,	Autoclaved
Olive oil	8 ,,	at 130°C
Salt mixture	5 ,,	f o r 5
Cod liver oil	2 ,,	hours
Dried yeast powder	5 ,,	

In diets 1, 2, 3, 4, 5, 6, 7 and 8 green vegetables = lettuce, cabbage and spinach in equal parts
root vegetables = potatoes and yams in equal parts

on common cereal staples Two foods not produced in India at present on a commercial scale—soya bean and dried skim milk—were included in certain of the diets, these foods are available at reasonable cost in the larger cities, and, given popular demand, could presumably be produced in India itself Condiments, which have little influence on cost or nutritive value, were not included in the diets Diets 1 to 9 were calculated to cost from Rs 4 to Rs 5 per month per adult man, on the basis of retail prices obtaining in the Madras Presidency Diet 10, which contains abundant milk, is somewhat costlier Diet 11 is that given to the stock rats at Coonoor, it contains more milk, in proportion to other foods, than is likely to be present in any human diet, and was included in the group to provide data as to the growth of our rats in optimum, or at least excellent, dietary circumstances Diet 12 is the vitamin-free basal diet commonly used in the laboratory, supplemented by cod-liver oil and dried yeast powder To investigate their nutritive value, the diets were fed to groups of young rats and subjected to chemical analysis

RAT GROWTH TESTS

Groups of young rats, 12 in each, of 35 g to 50 g weight, were fed the various test diets, *ad lib*, in the proportions indicated, for a period of 10 weeks Weight curves are given in Figs 1 and 2, and average weekly increase in weight in Table II —

TABLE II

Average weekly increase in weight during
10 weeks on test diets

Number of diet	Weekly increase in weight
1	7 4
2	6 9
3	8 0
4	8 4
5	8 2
6	9 8
7	8 2
8	7 6
9	7 9
10	8 7
11	10 6
12	8 7

CHEMICAL ANALYSIS

The diets were analysed for their content of protein, fat, carbohydrate, calcium, phosphorus iron, vitamin A, and carotene The results of chemical analysis are set out in Table III, the composition of diets 11 and 12 being given, for purposes of comparison, on a 2,700 calorie basis Vitamin A and carotene estimations were carried out by the spectrographic method described by De (1935)
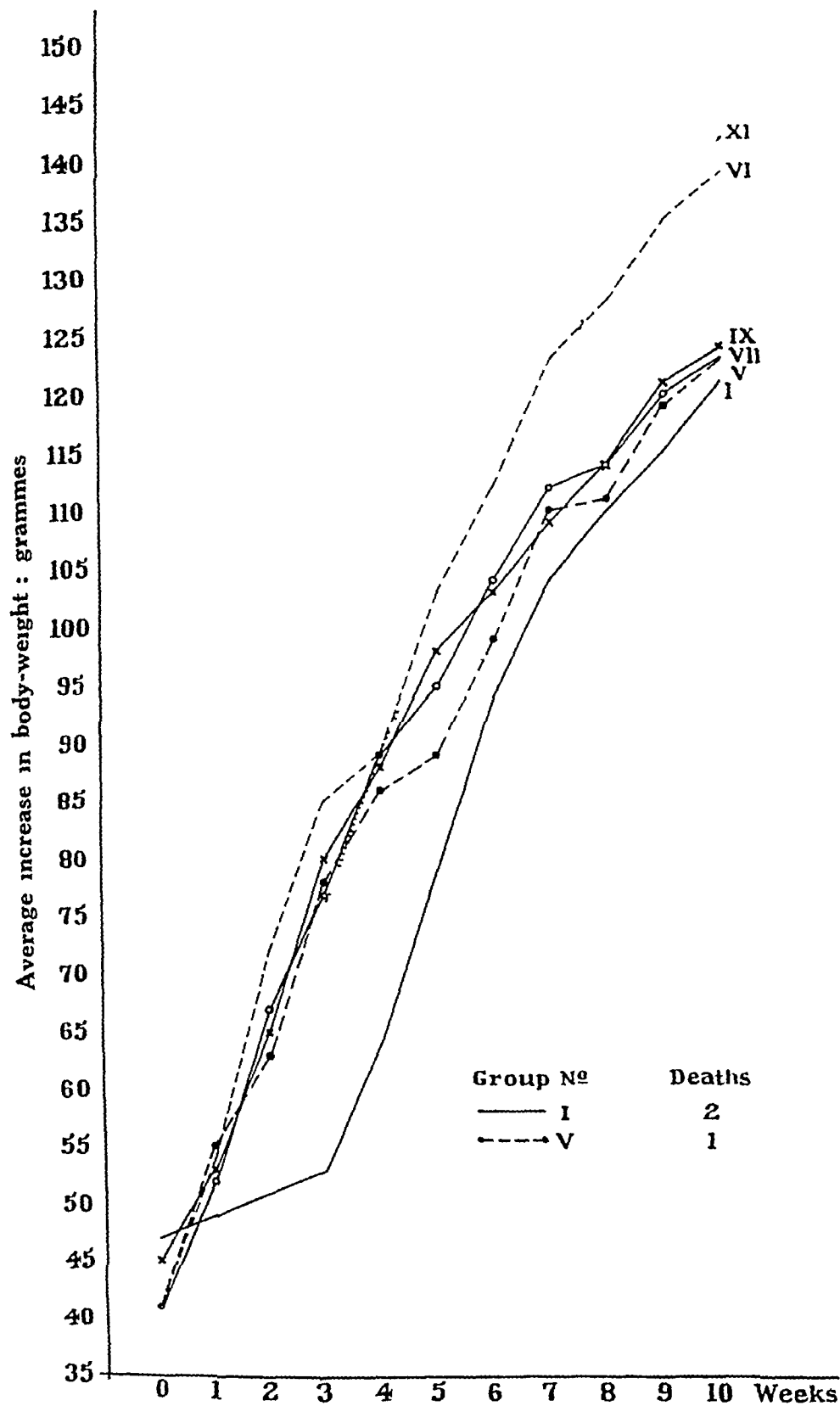
Fig 1. Showing average increase in weight and mortality on diets 1, 5, 6, 7, 9 and 11
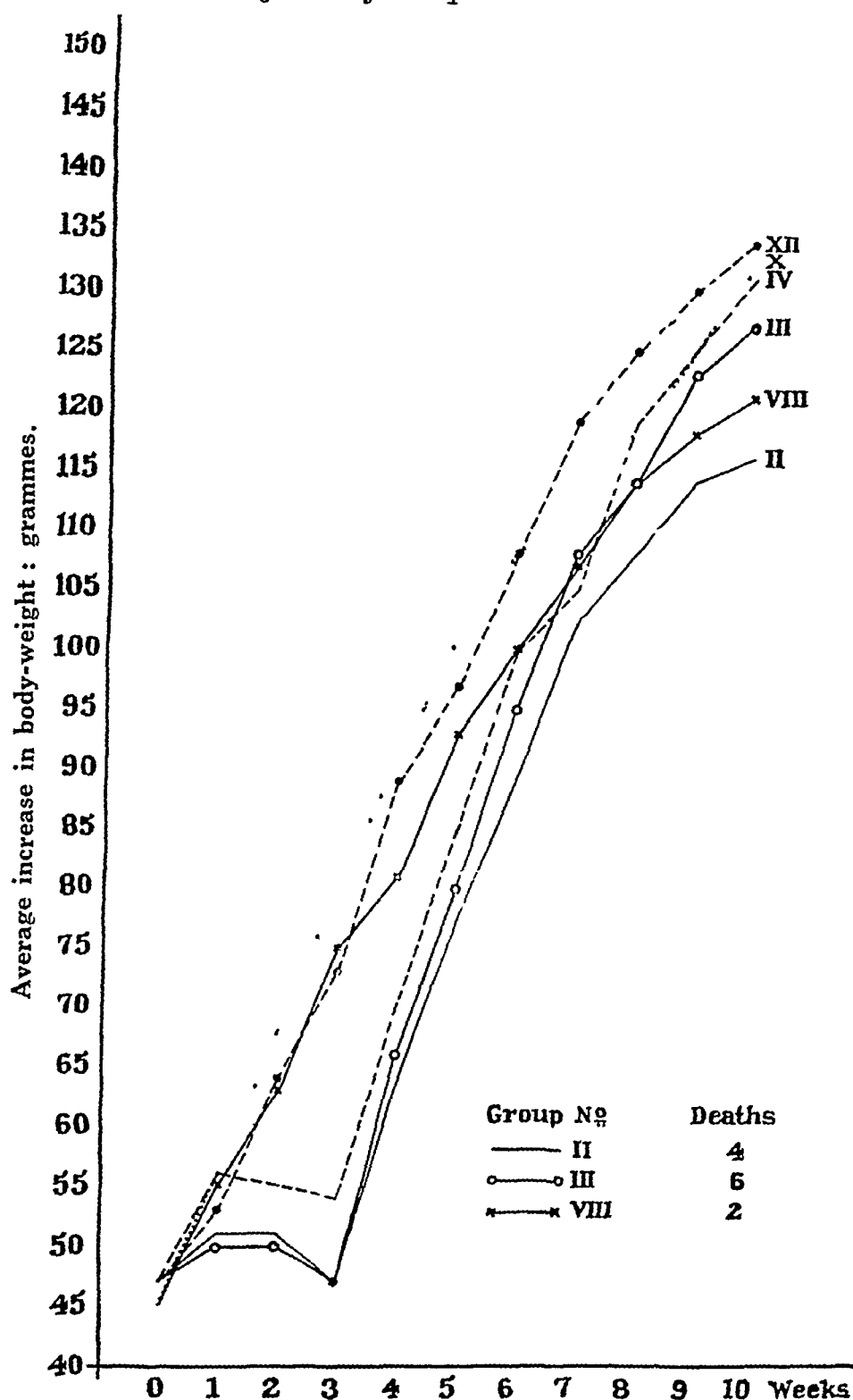
FIG 2   Showing average increase in weight and mortality on diets 2, 3, 4, 8, 10 and 12

TABLE III

Chemical composition of test diets

Diet number	Calories	Protein (grammes)	Fat (grammes)	Carbohydrate (grammes)	Calcium (grammes)	Phosphorus (grammes)	Iron (grammes)	Vitamin A* (γ)	Carotene (γ)
1	2,771	82 1	65 1	464 1	1 01	1 21	0 076		6,944
2	2,809	71 6	64 8	484 9	0 83	1 04	0 082		7,068
3	2,885	82 0	63 9	496 4	0 85	1 21	0 11ı		7,006
4	2,709	77 2	69 3	444 2	1 44	1 36	0 116		6,696
5	2,734	80 4	67 3	452 4	1 36	1 26	0 121		6,159
6	2,721	84 6	66 6	445 8	2 70	1 18	0 114		7,462
7	2,613	80 2	62 2	433 1	1 17	1 02	0 126		6,198
8	2,641	77 2	68 7	428 6	1 46	1 02	0 090		7,189
9	2,602	88 1	69 0	429 6	2 30	1 26	0 102		7,953
10	2,978	100 6	94 7	430 7	1 57	1 80	0 144	834	9,540
11	Per 2,700 calories	99 4	93 4	366 9	1 60	1 89	0 094	High vitamin A content	7,506
12		177 1	88 7	299 4	3 40	2 69	0 301	8,872	

* In diets 1 to 9 the only animal foods were dried skim milk and butter-milk, which are negligible sources of vitamin A Diet 10 contained whole milk, ghee and meat The vitamin A content of the stock diet (11) is high

DISCUSSION OF RESULTS

Rat test —On diets 1, 2, 3, 5 and 8 all the rats did not survive for the experimental period of 12 weeks. On diet 3 there were 6 deaths, on diet 2, 4 deaths, on diets 1 and 8, 2 deaths, and on diet 5, 1 death. Most of these were due to pneumonia and enteritis. Since we have observed that death from intercurrent infections rarely occurs among the animals fed on the stock diet (11), diets which were associated with such infections cannot be regarded as completely satisfactory. In the groups in which deaths occurred, the surviving (and presumably stronger) animals showed moderate growth. Average growth on all the ' cheap ' diets was less than that on the stock diet. The Coonoor rats are somewhat smaller than stocks commonly used in dietary studies in England and America, and average increase in weight even on the excellent stock diet is only 10 6 g per week.

The diets based on a combination of milled rice with cambu, whole-wheat flour and cholam respectively (1, 2 and 3) did not satisfactorily pass the biological test, although their protein and mineral content was raised by the addition of dried skim milk. Similarly, diet 8, in which the only cereal present was rice and the protein content was supplemented by a comparatively large addition of soya bean, did not support growth as efficiently as the stock diet, and two of the rats in this group died. The combination of rice with ragi (4) proved more satisfactory. These results suggest that it is difficult to devise a completely satisfactory diet, of which milled rice is the main ingredient, at the low cost of about Rs 5 per month. It is probable that if a diet based on rice, or rice in combination with cambu, wheat, or cholam, is to be completely adequate, more milk, green vegetables, etc, must be included than is present in diets 1, 2, 3 and 8.

Diet 6 in which ragi is combined with soya bean, produced excellent growth, and its chemical composition is satisfactory. This diet was calculated to cost a little over Rs 4 per month.

The calorie values of the diets as calculated on the basis of chemical analysis were in general agreement with estimates based on textbook figures. The proportion of protein in certain of the diets is low according to current standards, on the whole the diets less abundantly supplied with protein (and probably also with vitamin B_2) were those which were least satisfactory judged by the biological test. There is no reason to suppose that fat is present in inadequate amounts. The mineral content of the diets is high, when judged by Sherman's well-known standards. Vitamin A and carotene are probably present in sufficient abundance— the vitamin A activity of the cheap diets being 7 or 8 times that of a diet found to be associated with xerophthalmia (Aykroyd and Krishnan, 1936). Vitamin C requirements are probably covered by the leafy and root vegetables included in the test diets. In the uncooked state 8 ounces of green vegetables and 2 ounces of root vegetables of the kinds described would contain 150 mg to 300 mg of vitamin C (Ranganathan, 1935). If adult requirements of vitamin C are reckoned as 50 mg daily, a fair margin is left for losses on cooking.

In devising actual dietary schedules and specimen menus on the basis of these and subsequent investigations, a certain amount of adjustment and replacement will be necessary. A calorie intake of 2,700 to 3,000 would probably meet the needs of average Indian men engaged on average work, at any rate as far as

Southern India is concerned, if necessary, calorie content can be raised by adding extra cereal, without much increase in cost   The energy requirements of women, and children of different ages, can be assessed by applying the international scale of family coefficients *, or some other suitable scale, such as Cathcart's (1931)

Our diets were originally conceived as suitable for adult men   they were, however, tested by being fed to growing animals   Generally speaking, the relative cost of adequate diets for different age groups cannot satisfactorily be deduced from a scale of energy requirements   It is usually held that children need more protein and mineral salts per kilo of body-weight than adults   More milk and less cereal and other foods means increased cost   In considering the cost of minimum adequate diets for children, and also cost per family, it would probably be advisable to assume that children's diet should contain more milk, in proportion to other foods than is included in our diets

Pulses (apart from soya bean) are roughly similar in price and food value and thus interchangeable in diet schedules   In the vegetable group considerable variation is possible   potatoes can be replaced by other root vegetables, spinach and cabbage by other green leafy vegetables etc   As far as our present knowledge goes, vegetable oils in common use in India do not vary greatly in nutritive value

These experiments may be regarded as a preliminary to devising suitable cheap ' well-balanced ' diets for human use   A number of other diets are at present being tested in a similar manner   But it is clear that, while chemical analysis and biological assay may provide useful information as to the value of various dietary combinations, the ultimate test must be their effect on human beings   The next step must be to ' try out ' in practice diets resembling those investigated in the laboratory and found satisfactory, e g , in boarding schools for children

SUMMARY

A number of diets, costing from Rs 4 to Rs 5 per adult man per month, and conforming in a general way with diets in habitual use in India, have been tested by rat growth experiments, and subjected to chemical analysis

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THE CAROTENE AND VITAMIN A REQUIREMENTS
OF CHILDREN

BY

W. R. AYKROYD

AND

B. G. KRISHNAN

(Nutrition Research Laboratories, I. R. F. A., Coonoor, S. India)

THE problem of the vitamin requirements of human beings is in process of reduction to a quantitative basis. A number of workers have attempted to estimate human requirements of vitamin A, B₁ C, and D in terms of biological rat units, International Units, and of the pure vitamin itself (Harris, 1933; Rose, 1933; Sherman, 1933; Salter, 1934; Szent-Gyorgyi, 1934; Cowgill, 1934; Harris and Ray, 1935). For the establishment of 'standards of necessary vitamin intake', it is useful to know that a certain intake is associated with deficiency symptoms; 'minimum' or 'optimum' requirements must exceed this intake. The present paper records the association of xerophthalmia in children with a known intake of carotene.

In the course of a visit to certain organized labour camps in the Bellary district of the Madras Presidency, it was observed that a number of children were suffering from 'smokiness' and dryness of the conjunctiva, while a number showed the yellowish foamy patches in the conjunctiva known as 'Bitot's spots'. Evidence of the existence of vitamin A deficiency in the camps was thereby provided, and a more systematic investigation was undertaken. With the help of an experienced ophthalmic surgeon, 436 children between the ages of 1 and 12 were examined for ocular signs suggestive of vitamin

A deficiency   The results of investigations carried out in 3 camps were as
follows —

TABLE 1

Incidence of xerophthalmia

Number of camp	Number of children examined	Cases showing eye changes associated with vitamin A deficiency	
		Total number positive	Percentage of number examined
1	62	22	35
2	125	28	23
3	249	66	27
Totals	436	116	27

The children examined were a random sample, and included all ages from 1 to
12   The majority of the positive cases were in the early stages of the disease
showing only dryness, 'smokiness', or wrinkling of the conjunctiva   About 30
per cent of positive cases, however, were in a more advanced stage, with definite
'Bitot's spots'   Doubtful cases were labelled negative

At the time of examination the children had been fed for 7 to 8 months on
the dietary given in Table II   Table III shows its carotene content, indicating
the amount supplied by each item of the diet —

TABLE II

Composition of camp diet as consumed daily by various age groups

Age group	Cholam (Andropogan sorghum) (grammes)	Dhal arhar (Cajanus indicus) (grammes)	Vegetables and condiments (grammes)
1–5	184	14	14
5 8	283	28	20
8–12	311	31	24

with vegetable oil in small quantities

TABLE III

Carotene content of camp diet

Age group	Cholam (g)	Carotene (γ)	Dhal arhar (g)	Carotene (γ)	Vegetables (g)	Carotene (γ)	Total carotene (γ)
1–5	184	386 4	14	31 2	14	37 2	454 8
5–8	283	594 3	28	62 3	20	53 0	709 6
8–12	311	653 1	31	68 5	24	63 6	785 2
					Average of 3 groups		683 2

The carotene content of a sample of cholam used in the camps was estimated in the laboratory by Mr N K De, by the spectrophotometric method recently described (De, 1935)   The carotene figures for dhal arhar are derived from the spectrographic assay of a sample of dhal arhar closely resembling that supplied to the children   In estimating the carotene content of the ' vegetable and condiment ' ration, it was assumed that the latter consisted of brinjal (*Melongina solanum*) and bitter gourd (*Momordica cherantia*) in equal quantities these being vegetables commonly used in the camps , here again, results obtained by spectrographic assay were used   The figures given in this column represent only a rough estimate since we have no exact knowledge of the composition of this part of the ration   But the total condiment and vegetable ration is so small that, whatever its ingredients, it cannot greatly influence total carotene intake   Average total intake would be increased by about 20γ if calculations were made on the assumption that vegetables very rich in carotene—e g , amaranth and cabbage—were invariably supplied

Carotene content per gramme of the various items in the diet is as follows —

Cholam	2 10 γ
Dhal arhar	2 20 ,,
Brinjal	0 05 ,,
Bitter gourd	5 25 ,,

It may be assumed that the diet was entirely devoid of vitamin A   Spectrographic assay has shown that cholam, dhal arhar, brinjal, and bitter gourd lack this factor   Vitamin A has not been found in a considerable series of vegetable foods investigated in this laboratory

INCIDENCE OF ' ANGULAR STOMATITIS '

The presence of ' angular stomatitis ' (i e , a raw fissure of about $\frac{1}{8}''$ to' $\frac{1}{4}''$ at the angles of the mouth) in the groups of children examined is of interest   Seventy-one children (16 per cent) showed this condition in various degrees   Some of those with eye signs had angular stomatitis also, but there was no definite association

of the two conditions   Further study of the possible association between ' angular stomatitis ' and vitamin A and carotene deficiency is required

No attempt was made to study the incidence of hæmeralopia or of other conditions which have been ascribed to vitamin A deficiency

DISCUSSION

Individual physiological variation may account for the fact that only a certain proportion of children in the camps showed positive symptoms   In any population group consuming the same defective diet, signs of deficiency appear earlier and are more pronounced in certain individuals than in others   On the other hand, the physical condition of the children on entering the camps must have shown considerable variation

The children observed were taken into the camps from a district suffering from famine, it may therefore be inferred that in general the diet previous to admission was extremely deficient in vitamin A activity   A larger daily intake of vitamin A or carotene would be required to cure symptoms of deficiency than to supply the daily needs of individuals whose livers are well stored with vitamin A   Further, Bauman, Rissing and Steenbock (1934) have shown that, in rats depleted of vitamin A, and subsequently given large amounts of the vitamin, liver storage of the factor was less efficient than in normal rats, a possible explanation being that vitamin A is poorly absorbed by depleted animals   It may, however, legitimately be argued that a diet which, after being consumed for 7 to 8 months, is found to be associated with xerophthalmia, is deficient in vitamin A activity, and that ' normal requirements ' of carotene are in excess of the amount contained in the diet

Moore (1933) reached the conclusion that equal weights of β carotene and pure vitamin A have the same biological activity   Carr and Jewell (1933) found that their richest vitamin A concentrate was 1 6 times more potent than pure carotene (i e, the 1932 International Standard Preparation, which is mainly β carotene but contains some α carotene)   On the other hand, the work of Booth, Kon and Gillam (1934) suggests that the vitamin A present in butter is 6 times as potent as the carotene in butter   According to Kuhn, Brockman et al (quoted by Gillam and Heilbrom, 1935) the α and γ forms of carotene are physiologically only half as active as the β isomeride   It is probable that the carotene in the vegetable diet described in the present paper was present in both the α and β forms, with excess of the latter

It is difficult to deduce from the present observations, which concern ' subminimal ' carotene intake, any convincing data as to ' sub-minimal ' amounts of vitamin A (*strictu sensu*) for human beings. We have no knowledge as to how far the carotene in the labour camps' diet was absorbed and utilised   According to Clausen (1933) carotene is less readily absorbed from the intestinal tract of children than is vitamin A   Drummond et al (1935) observed that, in a case of chylothorax, ' the absorption of vitamin A from the intestine was much more satisfactory than that of its precursor carotene '   It is unjustifiable to assume, on the basis of Carr and Jewell's (*loc cit*) biological assays with pure materials, that 700ν of carotene in a mixed vegetable diet would possess

the same vitamin A activity for human beings as 450γ of vitamin A also contained in a mixed diet

In the almost complete absence of quantitative data as to human needs of the vitamin A factor, the hypothesis that 700γ of carotene (roughly 1 200 International Units) represents an inadequate intake for children may be of use in practical dietetics In the light of the present observations, Salter's (*loc cit*) estimate that 0 3 mg (300γ) of carotene represents minimal daily normal requirements is too low Optimum human requirements of vitamin A activity should perhaps be set very considerably above the ' sub-minimal ' figure recorded in this paper—in the neighbourhood of 3–5,000 International Units ?

SUMMARY

1 Eye symptoms indicative of vitamin A deficiency were observed in 27 per cent of a group of 436 children aged between 1 and 12, in a number of labour camps

2 The diets consumed by age groups 1–5, 5–8, and 8–12, are estimated to contain about 454γ, 709γ, and 785γ of carotene, respectively

3 It follows that optimum requirements of carotene must exceed that of the diets described, and the establishment of a ' sub-minimal ' figure of carotene intake, associated with deficiency symptoms, may be of use in practice

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OBSERVATIONS ON THE HEART RATE IN VITAMIN B_1 AND C DEFICIENCY.

BY

G. SANKARAN

AND

B G KRISHNAN

(*Nutrition Research Laboratories, I R F A , Coonoor, S India*)

DRURY *et al* (1930) reported that bradycardia was a regular symptom of vitamin B_1 deficiency in pigeons and rats, and that administration of vitamin B_1 concentrates cured this symptom   Birch and Harris (1934) made use of this fact to elaborate a technique for estimating the vitamin B_1 content of food-stuffs, the rat being the experimental animal   As the method described is less time consuming than most other biological methods used for vitamin B_1 estimations, a study of it was undertaken in these laboratories   Our first step was to obtain electrocardiographic records of heart rate in pigeons and rats, fed on complete and vitamin B_1 deficient diets

METHOD

The cardiograph used was a portable Matthew's inkwriter electrocardiograph which, as the name indicates, makes a record in ink on a reel of paper   The makers provide a special celluloid transparent time scale for reading the record   The electrodes are hypodermic needles electrically connected to the lead wires of the cardiograph, and are inserted under the skin of the animal   The pigeon is laid on its back and two sand bags are laid across the outstretched wings while another is placed over the legs   Two needles are inserted under the lower surface of the wings close to the body, while a third is inserted under the skin of the left thigh Tracings of all the three leads were obtained but usually one lead—as a rule the second—was convenient for study   While the cardiograph is being taken precautions are taken to have the animal perfectly still   No anæsthetic was found necessary   The rat was conveniently secured on its back on a wooden board by string attachments tied to each of its four paws while the head is fixed by hooking up the incisor teeth   This simple method of fixing was quite efficient and no clamps were needed as suggested by Drury and Harris   For the rats the leads were inserted one under the right leg and the other, which corresponds to the second lead, under the left chest wall   The record is taken when the animal is still

<center>THE DIETS</center>

The control pigeons were given mixed grains and the vitamin B$_1$ deficient pigeons washed polished raw rice   The control rats were given the laboratory stock diet consisting of wheat *chapatties* smeared with butter, fresh raw cabbage, raw carrots, sprouted Bengal gram and fresh milk (5 0 c c per rat) every day Lean meat was given twice a week   The vitamin B$_1$ deficient diet was the same as that described by Harris (1934)   sugar 60 parts, arachis oil 15, casein 20, salt mixture 5, autoclaved marmite 6, and cod-liver oil one drop per rat per day The marmite was titrated with glass electrode and alkali to pH 10 and then autoclaved at 130°C for one hour

<center>RESULTS</center>

Pigeons —Of the four pigeons in the deficient group three died within four weeks of being put on the experimental diet   All four developed 'polyneuritic' symptoms   Bradycardia was present in two deficient animals, the heart rate being from 185 to 285   The rate in four control pigeons fed mixed grains varied between 300 and 450   Large variations were observed in the heart rate of a group of four animals fed on washed polished raw rice with the addition of 20 mg of the vitamin B$_1$ International Standard Preparation

Rats —Two groups, each consisting of 24 animals, were used for the experiment Ten deficient animals developed paralysis of the hind limbs in about 6 to 7 weeks All the group which survived longer than 2 weeks showed a progressive drop in the heart rate   The heart rate in the animals fed on the stock diet remained about 450 to 550 throughout a period of 7 weeks   In the deficient animals the rate dropped to about 225 to 270 in 4 to 7 weeks   One animal showed a rate as low as 75 and another 150   These two were moribund at the time of taking the record Two of the animals died within two weeks   When the animals with a heart rate of about 250 were given foods containing vitamin B$_1$, such as sprouted gram or *chapatties*, they recovered their normal heart rate   It was observed that the heart rate did not drop in all cases when paralysis had set in   There were cases in which an animal showed paralysis and the heart rate did not drop until a few days after the appearance of paralysis   In some animals which showed no paralytic symptoms a low heart rate and heart block were observed   The figures of heart rate are given in Tables I and II   A few representative cardiographs of animals—showing the normal cardiograph, brady-cardia and heart block—are given in Fig 1

<center>VITAMIN C DEFICIENCY</center>

Guinea-pigs were used as the experimental animals   The scorbutic diet consisted of crushed oats and 50 c c of autoclaved milk per animal per day, and the control diet consisted of cabbage, carrots, sprouted Bengal gram, and rice bran   Twelve animals were put on each diet and cardiograph tracings of each animal secured every week for a period of 84 days   During this period only two animals in the vitamin C deficient group died, both within 35 days To prevent the animals dying off rapidly, a little cabbage was given to every animal that appeared very sick

Rat No 6252　Normal　Heart rate, 510
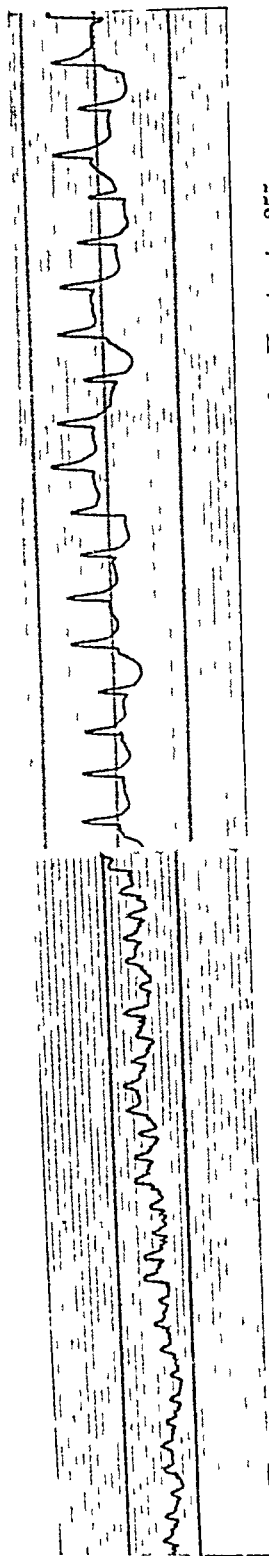
Rat No 6233　Bradycardia　Heart rate, 255

Rat No 6225　Bradycardia　Heart rate, 225
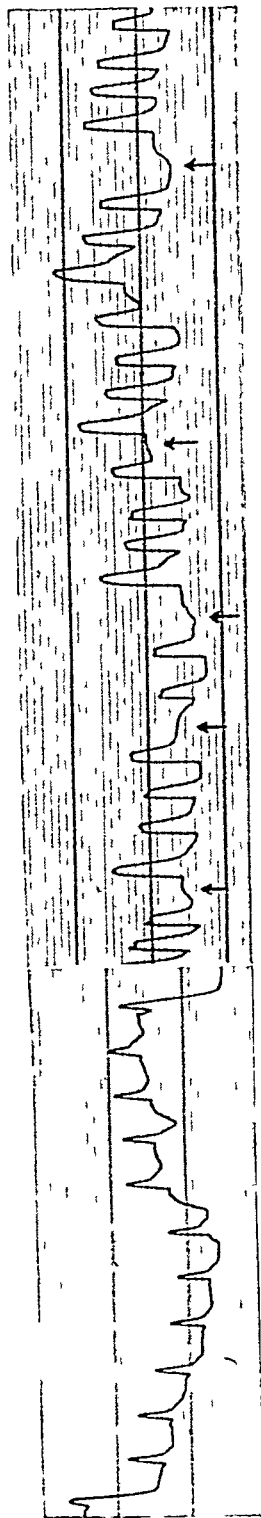
Rat No. 6219　Heart block.　Heart rate, 270
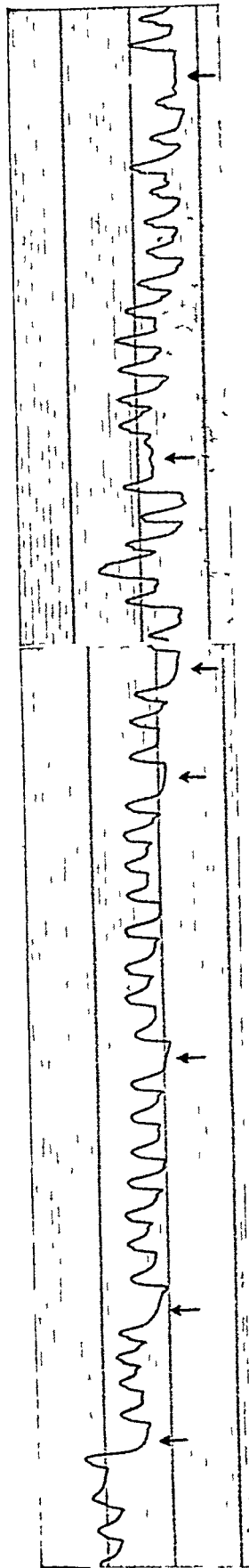
Rat No 6231　Heart block　Heart rate, 285

Rat No 6225　Heart block　Heart rate, 300

Fig 1
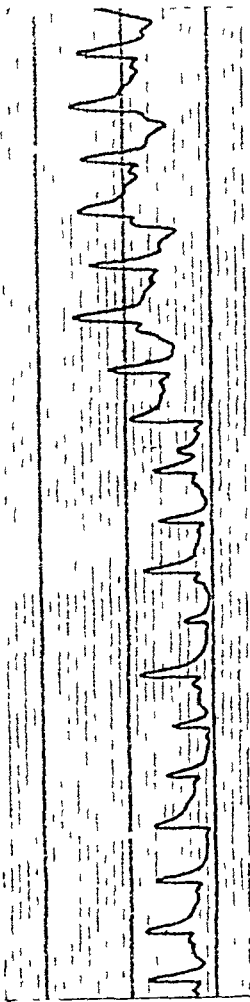
Guinea pig No 12   Normal   Heart rate, 255.
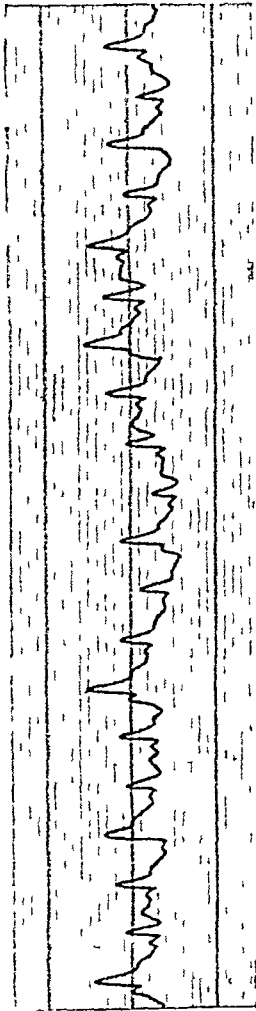
Guinea-pig No 16   Normal   Heart rate, 255.
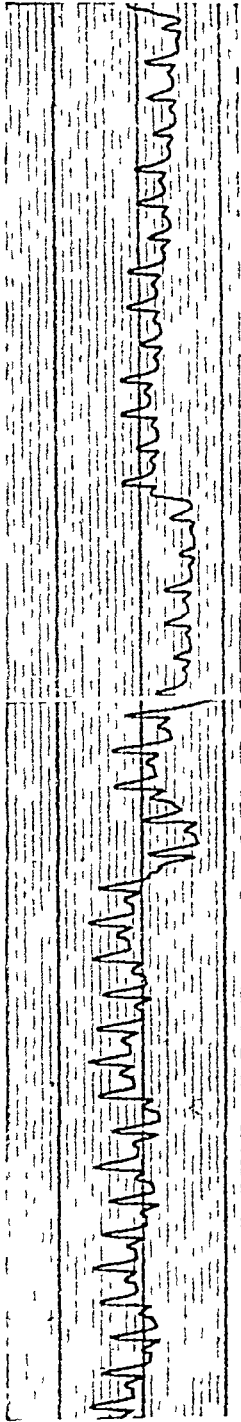
Guinea pig No 28   Tachycardia.   Heart rate, 360.

Guinea-pig No 31   Tachycardia   Heart rate, 375.

Fig 2

RESULTS

The average heart rate of normal guinea-pigs was found to be between 250 and 275 per minute   Animals on a scorbutic diet showed a marked increase in the heart rate to as much as 300 to 375   The increase was found to begin after the ninth week   This result is in accord with the clinical observation that tachycardia occurs in human scurvy   Table III gives the data obtained   The cardiographs of a guinea-pig and of a scorbutic animal are appended in Fig 2

TABLE I.

Heart rate in rats

STOCK DIET

Experiment commenced on 10–8–34.

Rat number	18–8–34	24–8–34	4–9–34	10–9–34	17–9–34	24–9–34	2–10–34
6239	480	525	435				450
6240	570	435	465				435
6241	465	450	480				540
6242	600	465	465				495
6243	480	600	465				435
6244	510	495	495				570
6245	510	495	510				
6246	450	540	585				570
6247	510	525	510				
6248	555	600	510				495
6249	525	510	435				525
6250	540	510	540				465
6251	510	495	450				405
6252	510	480	465				405
6253	510	465	480				480
6254	480	480	465				570
6255	510	465	435				435
6256	570	465	495				435
6257	600	480	525				510
6258	495	450	435				525
6259	540	465	525				510
6260	540	495	495				570
6261	480	450	435				450
6262	480	495	450				495

TABLE II

Heart rate in rats

VITAMIN B₁ DEFICIENT DIET

Experiment commenced on 10-8-34

Rat number	18-8-34	24-8-34	4-9-34	10-9-34	17-9-34	24-9-34	2-10-34
6215	615	570	540	525	525	315	210
6216	555	540	75				.
6217	570	555	450	300			
6218	510	510	420	420	345	270	
6219	540	510	435	330	270	270	270
6220	435	540	450	360	345	300	240
6221	450	480	450	390	390	285	255
6222	540	480	450	480	430	300	270
6223	510	570	510	374	465	210	465
6224	525	525	450	450	450	210	525
6225	510	420	300	390	225		.
6226	570	510	555	405	375	300	255
6227	585	510	495	435	435	315	285
6228	570	480	420	420	150		
6229	510	510	480	465	420	270	
6230	600	540	510	465	285		..
6231	555	480	285	390	Cured		
6232	585	450	480	405	390	330	285
6233	510	405	420	255	450	Cured	
6234	510	525	510	435	345	315	255
6235	525	510	450	420	435	345	300
6236	555	525	480	435	345	285	222
6237	**540**						
6238	540						

TABLE III

Heart rate in guinea-pigs on control and scorbutic diets

Experiment commenced on 24–9–34

CONTROL DIET

Animal number	22-10-34	29-10-34	5-11-34	12-11-34	19-11-34	26-11-34	3-12-34	10-12-34	17-12-34
10	345	345	345	315	330	345	345	285	270
11	285	300	330	285	330	300	315	270	285
12	360	330	315	330	315	285	285	255	240
13	255	245	300	255	315	255	285	255	285
14	315	345	315	265	300	265	240	250	245
15	225	270	330	280	290	230	285	210	225
16	180	210	190	230	285	250	270	255	266
17	330	315	345	320	345	345	270	240	230
18	240	285	320	285	285	215	315	285	285
19	210	255	300	285	315	275	270	240	255
20	285	270	285	255	285	255	240	215	225
21	240	300	320	315	285	285	255	255	270
TOTALS	3,270	3,470	3,695	3,420	3,680	3,305	3,275	3,015	3,080
AVERAGES	273	289	308	285	307	275	273	251	257

TABLE III—concld

SCORBUTIC DIET

Animal number	22-10-34	29-10-34	5-11-34	12-11-34	19-11-34	26-11-34	3-12-34	10-12-34	17-12-34
22	300	300	285	255	270	270	315	330	335
23	285	270	315	290	330	305	315	330	335
24	180	240	240	240	250	240	285	305	300
25	315	300							
26	240	255	240	240	255	225	285	330	330
27	330	330	300	270	300	300	335	345	375
28	225	375	345	270	300	315	360	360	360
29	345	360	350	330	330	290	335	345	345
30	270	315	270	315	285	240	285	315	315
31	330	315	300	305	305	315	345	360	375
32	285								
33	375	335	315	360	330	270	300	330	330
TOTALS	3,510	3,455	2,960	2,875	2,955	2,770	3,160	3,350	3,400
AVERAGES	293	314	296	288	296	277	316	335	340

CONCLUSIONS

(1) Bradycardia was observed in two pigeons on a diet of polished rice

(2) Rats fed on a vitamin B₁ deficient diet consistently show a drop in the heart rate, which rapidly returns to normal when foods rich in vitamin B₁ are given The results obtained confirm the work of Drury and Harris

(3) Guinea-pigs on a vitamin C deficient diet develop tachycardia

Our thanks are due to Major-General Sir Robert McCarrison, under whose direction this work was done

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FURTHER STUDIES ON THE EFFECT OF STORAGE ON THE VITAMIN C POTENCY OF FOOD-STUFFS.

BY

S RANGANATHAN

(From the Nutrition Research Laboratories, I R F A, Pasteur Institute, Coonoor)

PREVIOUS work on the effect of storage on the vitamin C potency of food-stuffs has shown that a leafy vegetable, like spinach, rapidly loses its vitamin C on storage, while a fruit, like orange, loses little or none of it during storage for as long as two weeks (Ranganathan, 1935)   To obtain further data on this important question, investigations were extended to six more food-stuffs three leafy vegetables, coriander (*Coriandrum sativum*), tender amaranth (*Amaranthus gangeticus*) and fenugreek (*Trigonella fœnumgræcum*), and three others of a ' non-leafy ' nature, chillies (*Capsicum annuum*), mango and bitter gourd (*Momordica charantia*)

The experimental procedure adopted was much the same as that reported in the previous paper   In addition to determining the loss of vitamin C consequent on storage at room temperature in Coonoor, a hill station situated at about 6,000 feet above mean sea-level (the room temperature ranged during these experiments from 19°C to 22°C), the loss in vitamin C on storage at a slightly higher temperature, about equal to that obtaining in the plains during hot weather, was also investigated in the present series of experiments   Vitamin C was estimated by the chemical method described in the previous paper

LEAFY VEGETABLES

A fair quantity of each leafy vegetable was obtained in the fresh state and the leaves freed as far as possible of the adhering soil and water particles   Where the specimen could not be had fresh, it was obtained from the local bazaar, care be ng taken to obtain the best sample available   A representative sample of the food-stuff was then divided into several small fractions of equal weight—usually 5 or 10 gramme fractions—and preserved in paper packets, half of these at

room temperature and the remaining half in an incubator maintained at about 38°C   One of the fractions was immediately used for determining the initial vitamin C content and the rest investigated one by one after storage for stated intervals   The loss in weight of the specimens due to the evaporation of moisture on storage at room temperature and at the higher incubator temperature was also determined   The results of experiments with the six food-stuffs are set out in the accompanying tables —

TABLE I

Showing the effect of storage on the vitamin C potency of coriander leaves

The specimen was obtained from the local bazaar , initial vitamin C content=69 0 mg per cent

	STORAGE AT					
	ROOM TEMPERATURE (19°C TO 22°C)			38°C		
Period of storage	Vitamin C content in mg per cent of original weight	Percentage loss of vitamin C during storage	Percentage loss in weight due to loss of moisture	Vitamin C content in mg per cent of original weight	Percentage loss of vitamin C during storage	Percentage loss in weight due to loss of moisture
1	2	3	4	5	6	7
24 hours	28 5	58 7	57 0	22 0	68 1	61 2
30 „	19 5	71 6	59 4	17 0	75 4	64 2
48 „	16 3	76 4	75 8	13 0	81 2	80 2
54 „	12 0	82 6	82 8	10 0	85 5	86 0
72 „	6 5	90 6	84 0	6 5	90 6	86 4
96 „ ••	6 1	91 2	84 6	6 0	91 3	86 6
102 „	5 8	91 6	85 4	5 8	91 6	86 6
120 „	5 5	92 0	85 4	6 0	91 3	86 6
144 „	4 8	93 0	85 4	5 0	92 7	86 6

TABLE II

Showing the effect of storage on the vitamin C potency of tender amaranth leaves

Specimen obtained from the local bazaar , initial vitamin C content=112 1 mg per cent

Period of storage	STORAGE AT					
	ROOM TEMPERATURE (19°C TO 22°C)			38°C		
	Vitamin C content in mg per cent of original weight	Percentage loss of vitamin C during storage	Percentage loss in weight due to loss of moisture	Vitamin C content in mg per cent of original weight	Percentage loss of vitamin C during storage	Percentage loss in weight due to loss of moisture
1	2	3	4	5	6	7
24 hours	59 9	46 6	34 0	27 2	75 7	70 4
43 ,,	29 8	73 4	46 0	10 0	91 1	85 0
48 ,,	25 6	77 2	49 8	4 5	96 0	85 8
67 ,,	13 1	87 4	57 8	4 5	96 0	86 2
72 ,,	12 2	89 1	76 8	4 5	96 0	86 8
91 ,,	9 3	91 7	79 0	4 5	96 0	86 8
96 ,,	5 5	95 1	80 4	4 5	96 0	86 8

TABLE III

Showing the effect of storage on the vitamin C potency of fenugreek leaves

Specimen obtained from a garden close by, initial vitamin C
content = 140 7 mg per cent

	STORAGE AT					
	ROOM TEMPERATURE (19°C TO 22°C)			38°C		
Period of storage	Vitamin C content in mg per cent of original weight	Percentage loss of vitamin C during storage	Percentage loss in weight due to loss of moisture	Vitamin C content in mg per cent of original weight	Percentage loss of vitamin C during storage	Percentage loss in weight due to loss of moisture
1	2	3	4	5	6	7
5 hours	136 7	2 8	16 9	129 4	8 0	22 0
24 ,,	98 9	29 7	46 1	68 4	51 4	50 6
29 ,,	86 9	38 2	52 3	53 8	61 7	57 1
48 ,,	74 0	47 4	73 2	34 8	75 3	80 2
53 ,,	61 1	56 6	75 7	30 5	78 3	81 8
72 ,,	33 5	76 2	83 5	11 6	91 8	88 2
96 ,,	12 9	90 8	86 8	12 5	90 8	89 5
101 ,,	13 9	90 1	87 1	9 5	93 2	90 4
120 ,,	13 4	90 5	89 1	10 6	92 5	90 4

In the above experiments with leafy vegetables, specimens of like weight were weighed out at the commencement ; they were then stored and examined at stated intervals for their vitamin C content   Hence, in calculating the results of columns 2 and 5 in Tables I, II and III, account was not taken of the loss in weight due to loss of moisture occurring during storage   The loss of vitamin C on storage recorded above represents the extent to which a known weight of leafy vegetable loses its vitamin C on storage, irrespective of the loss in moisture occurring at the same time   But, on the practical side, it may be argued that the consumer is more likely to be concerned with the vitamin C content of the sample he is purchasing no matter for how many hours or even days it has lain in the market   This aspect of the problem would assume importance only when the material is purchased by weight and when it loses weight considerably on storage ; it is not of moment with most leafy vegetables, which are usually purchased in bunches and not by weight   Hence, in the case of the leafy vegetables reported above, the loss in vitamin C expressed in terms of the original weight of the sample is of more practical significance than the loss calculated in terms of the existing weight of the material

at the various stages of storage   Calculations on the latter basis often lead to ano-malous results , values in excess of that contained in the fresh sample would be obtained for an absolutely dry specimen of the same   Not infrequently, the vitamin C content would appear to increase as the material stored gets drier and drier , further, while a leafy vegetable stored even for a few hours could be easily demonstrated to have lost a part of its vitamin C, the same material when perfectly dry after nearly a week's storage would be reported as containing more vitamin C than the original sample itself

Specimens of leafy vegetables preserved with stalk and root were found to lose vitamin C on storage at about the same rate as those separated from the stalk

MANGOES, CHILLIES AND BITTER GOURD

In the case of these foods, there was no purpose in testing the vitamin C potency of fragments of the same original specimen stored for various' periods, since in practice they are stored in an unbroken condition   It was therefore necessary to carry out estimations on different specimens which appeared to be of the same degree of freshness and ripeness at the beginning of the period of storage

Mangoes —A dozen fruits, of approximately the same size and degree of ripeness, were obtained from the same tree, through the courtesy of the Curator, Government Botanical Gardens, Ootacamund   The fruits were quite mature, but not fully ripe   Owing to unavoidable reasons, experiments on these fruits could not be started as soon as they arrived , they were therefore stored in the cold room at about 0°C for a little over a fortnight   They were then preserved, some at room temperature and some at 38°C   Vitamin C was estimated in the whole fruit, pulp, and outer skin of the fruits, and the results recorded in Table IV —

TABLE IV

Showing the effect of storage on the vitamin C potency of mangoes

Specimen obtained from the Government Botanical Gardens, Burliyar, Nilgiris

| | | STORAGE AT | | | | |
| | | ROOM TEMPERATURE (19°C TO 22°C) | | | 38°C | | |
Period of storage		Entire fruit vitamin C in mg per cent	Outer skin only vitamin C in mg per cent	Pulp only vitamin C in mg per cent	Entire fruit vitamin C in mg per cent	Outer skin only vitamin C in mg per cent	Pulp only vitamin C in mg per cent
	Nil	27 5	82 1	20 9	27 5	82 1	20 9
19	hours	26 7	86 2	17 5	30 8	89 4	16 4
48	,,	17 9	77 1	20 9	23 9	82 0	22 3
72	,,	23 9	90 7	17 6	*22 8	67 2	12 4
116	,,	*25 8	87 8	10 5	†7 1	5 0	7 6
154	,,	*16 2	47 4	9 7	†3 4	5 9	1 7
178	,,	‡11 3	26 9	7 1			

* Partly ripe   † A major portion of the fruit had decayed   ‡ Fully ripe

TABLE V

Showing the effect of storage on the vitamin C potency of chillies

Specimen obtained from the local bazaar

Period of storage	STORAGE AT			
	ROOM TEMPERATURE (19°C TO 22°C)		38°C	
	Vitamin C content in mg per cent	Percentage loss in weight due to loss of moisture	Vitamin C content in mg per cent	Percentage loss in weight due to loss of moisture
Nil	57 4		57 4	
19 hours	48 2	10 4	30 8	13 0
25 ,,	35 5	13 6	26 4	18 0
43 ,,	*21 0	22 8	*16 2	32 2
49 ,,	*16 6	23 8	*7 4	40 2

* Partly ripe

[All specimens preserved for over 49 hours were completely ripe, those preserved at 38°C for 43 hours and over were ripe The ripe ones gave on acetic acid extraction a reddish-coloured extract, and hence they could not be made use of for the estimation of vitamin C by the chemical method In the partly ripe chillies, the comparatively greener portion was used for analysis]

TABLE VI

Showing the effect of storage on the vitamin C potency of bitter gourd

Specimen obtained from the local bazaar

Period of storage	STORAGE AT					
	ROOM TEMPERATURE (19°C TO 22°C)			38°C		
	Vitamin C content in mg per cent	Percentage loss in weight due to loss of moisture	REMARKS	Vitamin C content in mg per cent	Percentage loss in weight due to loss of moisture	REMARKS
Nil	106 7			106 7		
24 hours	87 6	5 3	Quite green	72 2	13 9	Quite green

TABLE VI—*concld*

Period of storage	STORAGE AT					
	ROOM TEMPERATURE (19°C TO 22°C)			38°C		
	Vitamin C content in mg per cent	Percentage loss in weight due to loss of moisture	REMARKS	Vitamin C content in mg per cent	Percentage loss in weight due to loss of moisture	REMARKS
43 hours	75 4	9 7	Quite green	68 6	23 6	Slightly ripe, only the green portion was used for analysis
48 „	73 1	11 0	do	64 5	26 0	do
67 „	70 8	16 6	do	40 9	36 2	Fairly ripe
72 „	68 1	18 2	Just showing signs of ripening	35 9	39 9	do
91 „	64 5	24 5	do	25 4	53 2	Quite ripe
96 „	23 3	29 2	Quite ripe	12 7	58 2	Fully ripe, the fruit has burst open

It will be seen from the above tables that the loss of vitamin C in leafy vegetables on storage is quite considerable   Nearly three-fourths is lost on storage at 100m temperature for a period of 72 hours , the loss is greater still on storage at the higher temperature   A small amount of vitamin C persists in all the leafy vegetables examined, even after they have become absolutely dry , this amount ranges from 5 mg to 10 mg per cent   An approximate parallelism is noticeable between the loss of vitamin C on storage and the loss in weight due to loss of moisture occurring at the same time

The case of mangoes, chillies and bitter gourd is, however, different , the loss in vitamin C on storage is comparatively little even though one could not strictly deduce the percentage loss of vitamin C on storage, as was done with the leafy vegetables   Chillies do not lose vitamin C to the same extent as leafy vegetables as long as they remain green throughout   The vitamin C of mangoes is not appreciably lost on storage, until they completely ripen   The outer skin of the mango is fairly rich in vitamin C and continues so, as long as the skin remains green   Once it assumes a golden yellow or brownish hue, a sudden fall in its vitamin C content is observed , subsequent storage is accompanied by a further loss of vitamin C   The inside pulp conserves its vitamin C as long as the outer skin continues to be green , when the fruit ripens, the pulp too loses its vitamin C on storage   Storage

at the higher temperature hastens ripening as also the rate of destruction of vitamin C after ripening   Bitter gourd behaves likewise , as long as it remains green throughout, no appreciable loss of vitamin C occurs on storage   Once the outer skin becomes brown, indicative of ripening which is often accompanied by the bursting open of the fruit, there is a sudden fall in its vitamin C content   Even in the partly ripe bitter gourd, the comparatively greener portion contains more vitamin C than the browner portion

SUMMARY

(1) The effect of storage on the vitamin C potency of six food-stuffs, coriander, tender amaranth and fenugreek leaves, and mango, chillies and bitter gourd, was investigated.

(2) The green leafy vegetables—coriander, tender amaranth and fenugreek leaves—were found to lose vitamin C rapidly on storage, the loss being quicker at 38°C than at room temperature   The previous finding that leafy vegetables lose considerably more vitamin C on storage than other vegetables of a non leafy nature, and fruits, was confirmed

(3) As long as mangoes, chillies and bitter gourds remain green, the loss of vitamin C on storage is practically little , storage after ripening is accompanied by an appreciable loss of vitamin C

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BIOLOGICAL ASSAY OF VITAMIN A IN THE DIET OF INDIANS *

BY

ELLA SURIE, M SC (Lond),

Bedford College, University of London, and Lady Hardinge Medical College, New Delhi

MANY of the investigations into the problems of nutrition during the last two decades have centred round a study of the deficiency diseases   Numerous articles by medical and scientific authors describe such diseases experimentally produced in animals (McCarrison, 1921 , Funk, 1922 , Mellanby and Green, 1928, 1931 , M R C , 1932 , Mellanby, 1935), their early recognition (Wolbach and Howe, 1925 , Pillat, 1929 , Eusterman and Wilbur, 1932 , Thatcher and Sure, 1932 , Fox, 1932 , Jeans and Zentmire, 1934), their clinical symptoms, their cure by specific vitamins (Mellanby and Green, 1930, 1933) and how much of the concomitant disease and suffering may be prevented (Wright, 1931 , Kirwan, 1931 , McCarrison, 1932–33)

In Asia (Mori, 1904) vitamin A deficiency diseases have a much higher rate of incidence than in Europe and North America, but in times of stress, such as during and after the Great War, they were also widely prevalent in Denmark and Central Europe

Following up the suggestion of Bloch (1920–21) in Denmark that ' the stunted growth and general ill health as well as xerophthalmia were caused by lack of " these substances " — ' these substances " in this context signifying vitamin A—Mellanby and Green (1928) brought forward evidence of the anti-infective properties of vitamin A, from observations on experimental animals and human infective conditions   A field for inquiry into the prophylactic treatment of cutaneous infections so prevalent in the East has been initiated by Loewenthal (1933) in an interesting communication on cutaneous manifestations occurring in a Uganda Jail and Mackay (1934) confirms his opinion when she states that susceptibility to skin infections are ' probably the first stages of vitamin A deficiency in infants '

The diet throughout India is lacking in vitamins and abundant in cereal, it is a thoroughly unbalanced diet   To quote McCarrison ' In India we have to deal with combinations of food faults rather than single food faults '

The urgency of the situation has led, within the last few years, to the assay of vitamin A in some of the food-stuffs of India, notably fish liver and body-oils (Bacharach, 1930 , Ghosh *et al* , 1934 , Datta and Bannerjee, 1934), ghee (Brahmachari, 1932 , Greval, 1933) and fruit (Guha and Chakravorty, 1932–33)

In the above-mentioned investigations a recognized synthetic vitamin A free diet has constituted the experimental basal ration, but the investigations recorded in this communication have been carried out from a somewhat different angle that of correlating the effects of varying degrees of vitamin A insufficiency of

(*a*) an Indian dietary,

(*b*) an Indian dietary supplemented by known amounts of vitamin A containing foods,

with the severity of the lesions exhibited in the experimental animals

EXPERIMENTAL METHODS

The rate of reaction of the animals (albino rats) to the Indian dietary is slower than that to the standard synthetic basal ration generally fed   Time has therefore been sacrificed in order to feed a basal ration which approximates to the diet consumed by the inhabitants of India   The composition of this Indian diet is necessarily a compromise as there is no one diet common to the people of India This diet, synthesized from articles of food, not from pure food-stuffs, i e , proteins, fats, carbohydrates, etc , is not devoid of vitamin A, yet it is undoubtedly insufficient with respect to this vitamin   Its deficiency in vitamin D has been rectified by the administration of ' Radiostol '   In other respects the usual technique of the biological experiment has been observed

The rats were weighed twice weekly and the first appearance of xerophthalmia, abnormality of coat and inco-ordination of gait noted   At post-mortem a rough estimation of the amount of vitamin A in the liver was made, using the Carr-Price reaction (Carr and Price, 1926)   A Lovibond tintometer was not available , these results are, therefore, relative to one another

THE BASAL DIET

Rice (unpolished)	60 per cent.
Atta	30 ,,
Masoor dhal (*Lens esculenta*) or Muug dhal (*Phaseolus mungo*)	5 ,,
Linseed oil	4 ,,
Sodium chloride	1 ,,
Radiostol	0 5 unit per diem (3 5 units given twice weekly).

This will henceforth be called the ' Indian basal diet '

PREPARATION OF THE ' INDIAN BASAL DIET '

The food was cooked so as to resemble a human dietary as closely as possible [Incidentally according to Louveiro (1922) rats consume a cooked diet more readily than a raw diet]

Rice—plunged into boiling water and cooked till soft in an open pan

Atta—stirred into boiling rice and water when the rice was cooked

This treatment will rupture the starch grains and may be reckoned as equivalent to the amount of cooking when chapatties are made

Linseed oil—stirred into the rice-water-atta mixture

Dhal—boiled in water with the sodium chloride of the ration for about 2 to 3 hours in an open pan, then stirred into the above mixture

Sufficient food for 3, 4 or 7 days was prepared at a time and stored in the refrigerator The water content of the cooked diet was about twice that of the dry constituents, as this amount of water was ample, no additional water was given

SUPPLEMENTS TO THE ' INDIAN BASAL DIET '

Fresh cabbage	Green leaves (no stalks), these were chopped not minced
4 hours' boiled cabbage	Green leaves chopped Seven day ration boiled with water in an open dish for 4 hours Stored in refrigerator Cabbage and water fed
36 hours' heated cabbage	Green leaves heated 6 hours at 98°C to dry, subsequently heated for 36 hours at 120°C in an air oven to destroy carotene Amounts of boiled and heated cabbage fed as equivalents of fresh cabbage
Butter	Fresh cow butter
Ghee	Buffalo ghee

The rice, atta, dhal and ghee were obtained from distributors of Indian food-stuffs in London

RESULTS

Series I —25 rats (It was impossible to start the experimental diet until these rats were 60 days old The usual technique had therefore to be modified)

Total experimental period 103 days

The rats were fed on stock diet until the 44th day, after which they were given a vitamin deficient diet (Mellanby and Green, 1928) for 16 days, to deplete their vitamin A stores

Subsequently the rats were divided into three groups and fed as follows for a total experimental period of 103 days —

Group   1   10 rats 5 ♂ 5 ♀ 'Indian basal diet'

   „   2   9 „   4 ♂ 5 ♀   „   + 1 g 4 hours' boiled cabbage

   „   3   6 „   3 ♂ 3 ♀   „   + 1 g fresh cabbage

The results of this series are summarized in Table I, curves of average weights are shown in Chart 1 and photographs on Plate XXVII

*Series II —*30 rats   Total experimental period 140 days

All the rats were given the 'Indian basal diet' for 80 days, subsequently they were divided into 4 groups as follows for a further period of 60 days —

Group   1   8 rats 4 ♂ 4 ♀ 'Indian basal diet'

   „   2   8   „   4 ♂ 4 ♀   „   + 0 25 g 36 hours' heated cabbage

   „   3   8   „   4 ♂ 4 ♀   „   + 0 25 g 4 hours' boiled cabbage

   „   4   6   „   3 ♂ 3 ♀   „   + 0 25 g fresh cabbage

The results of this series are summarized in Table II and the curves of average weights are shown in Chart 2

*Series III —*21 rats   Total experimental period 105 days

All the rats were fed on the 'Indian basal diet' for 58 days, subsequently they were divided into 3 groups as follows for a further period of 47 days —

Group   1   7 rats 3 ♂ 4 ♀ 'Indian basal diet'

   „   1 later 3 „   1 ♂ 2 ♀ During the last 26 days of this period 2♂ and 2♀ (*see* group 4) were given butter in place of linseed oil in order to evaluate ghee relative to butter as well as to linseed oil

   „   2   7   „   4 ♂ 3 ♀ 'Indian basal diet' but 2 per cent linseed oil replaced by ghee

   „   3 .   7   „   4 ♂ 3 ♀ 'Indian basal diet' but all (i e , 4 per cent) linseed oil replaced by ghee

   „   4 (from group 1)
                  4 rats 2 ♂ 2 ♀ 'Indian basal diet' but linseed oil substituted by *butter* for last 26 days of experiment

The results of this series are summarized in Table III, and curves of average weights are shown in Chart 3

PLATE XXVII

At post-mortem

Just before post mortem

Fig A   Indian basal diet — xerophthalmia, stary coat, characteristic hunched attitude
  ,,   B   Indian basal diet + 10 g cabbage, boiled 4 hours, normal in appearance
  ,,   C   Indian basal diet + 10 g fresh cabbage, normal in appearance

TABLE I

Series I—'Indian basal diet' Cabbage Duration of experiment 103 days 25 rats

Diet and number of rats	Average gain in weight in grammes after diet changed	Incidence of xeroph thalmia	Incidence of cutaneous symptoms	Presence of vitamin A in liver as shown by Carr Price reaction for vitamin A	Post mortem notes	Other notes
Group 1 INDIAN BASAL DIET 10 rats {5 ♂, 5 ♀}	28 32	All exhibited varying degrees of xerophthalmia See Plate XXVII, fig A	Coats appeared 'stary' See Plate XXVII, fig A	All −	3 rats showed slight infection respiratory passages	1 ♂ died after 42 days. 1 ♀ died after 83 days. 1 ♂ moribund at termination of experiment. Abnormal resting position—gait awkward
Group 2 INDIAN BASAL DIET + 1 g 4 hours' boiled cabbage 9 rats {4 ♂, 5 ♀}	98 73	After 87 days one rat exhibited signs of xerophthalmia	After 92 days coats of 2 rats appeared 'stary'	8 rats slight reaction 1 −	N A D	Almost normal in appearance *See* Plate XXVII, fig B
Group 3 INDIAN BASAL DIET + 1 g fresh cabbage 6 rats {3 ♂, 3 ♀}	99 70	None *See* Plate XXVII, fig C	Beautiful glossy coats *See* Plate XXVII, fig C	All +	N A D.	Appeared to be normal rats

CHART 1.
Series I.
'Indian basal diet' 10 g cabbage 25 rats
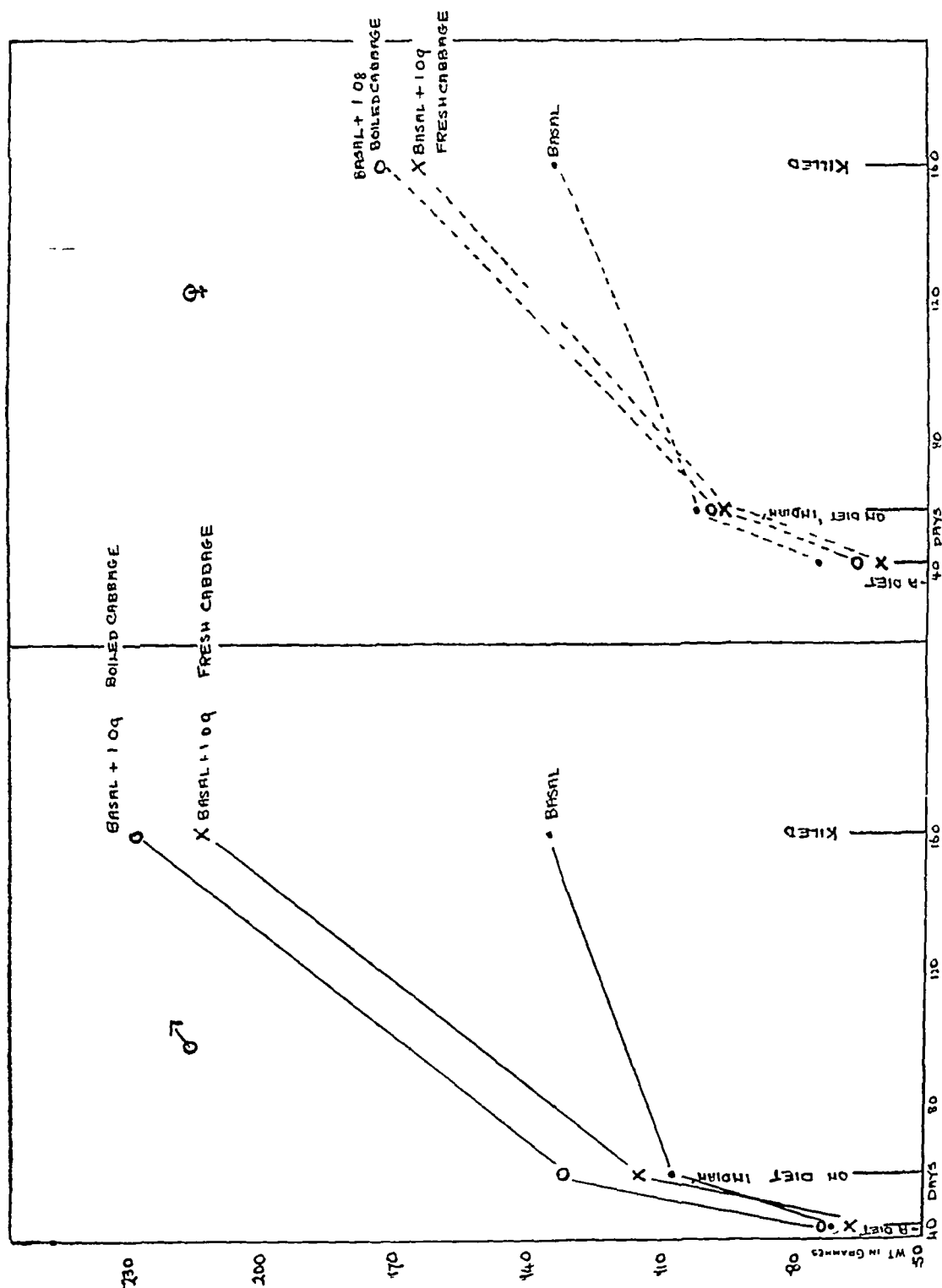
TABLE II.

Series II.—'Indian basal diet' Cabbage Duration of experiment 140 days 30 rats

Diet and number of rats	Average gain in weight in grammes after diet changed	Incidence of xerophthalmia	Incidence of cutaneous symptoms	Presence of vitamin A in liver as shown by Carr Price reaction for vitamin A	Post mortem notes	Other notes
Group 1 INDIAN BASAL DIET { 4♂ 4♀ } 8 rats	20 20	All exhibited xeroph thalmia	Coats 'starv'	All —	N A D	All rats exhibited signs of xerophthalmia before diet was changed. Healing occurred in groups 3 and 4
Group 2 INDIAN BASAL DIET + 0 25 g 36 hours' heated cabbage { 4♂ 4♀ } 8 rats	39 11	All exhibited xeroph thalmia	Coats 'starv'	All —	N A D	
Group 3 INDIAN BASAL DIET + 0 25 g 4 hours' boiled cabbage { 4♂ 4♀ } 8 rats	67 35	One rat exhibited xerophthalmia	Glossy coats	All —	N A D	
Group 4 INDIAN BASAL DIET + 0 25 g fresh cabbage { 3♂ 3♀ } 6 rats	56 40	None	Glossy coats	5 +, 1 very faintly +	N A D	

Chart 2.
Series II.

'Indian basal diet' 0.25 g cabbage   30 rats.
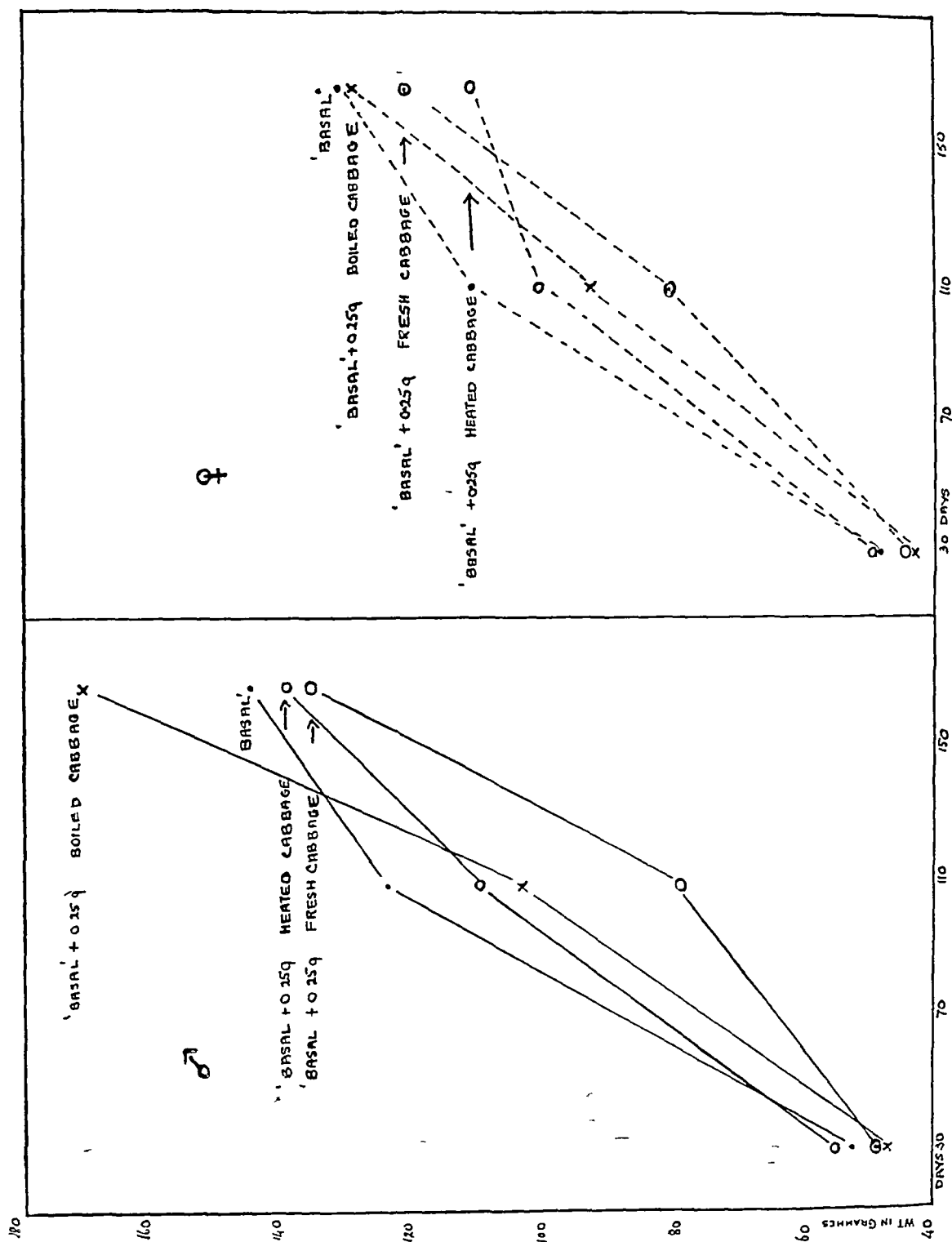
TABLE III.

Series III —'Indian basal diet'   Ghee   [Duration of experiment 105 days   20 rats

Diet and number of rats	Average gain in weight in grammes after change in diet	Incidence of xerophthalmia	Incidence of cutaneous symptoms	Presence of vitamin A in liver as shown by Carr Price reaction for vitamin A	Post mortem notes	Other notes
Group 1 INDIAN BASAL DIET 3 rats {1♂ 2♀}	14 25	—	Coats 'stary'	All —	N A D	7 rats originally in this group—4 rats used for group 4 experiment since reaction to this basal diet known from series I and II
Group 2 INDIAN BASAL DIET 2 per cent ghee 2 per cent linseed oil 7 rats {4♂ 3♀}	38 29	—	Not quite normal	All —	N A D	These two groups received the substituted diet for the whole subsequent period of 47 days
Group 3 INDIAN BASAL DIET Ghee 7 rats {4♂ 3♀}	44 27	—	Not quite normal	All —	N A D	
Group 4 INDIAN BASAL DIET Butter 4 rats {2♂ 2♀}	48 39	—	Improved	All —	N A D	This group formed part of group 1 till 79th day of experiment Substituted diet for last 26 days only

CHART 3
Series III
'Indian basal diet'   Ghee butter   21 rats
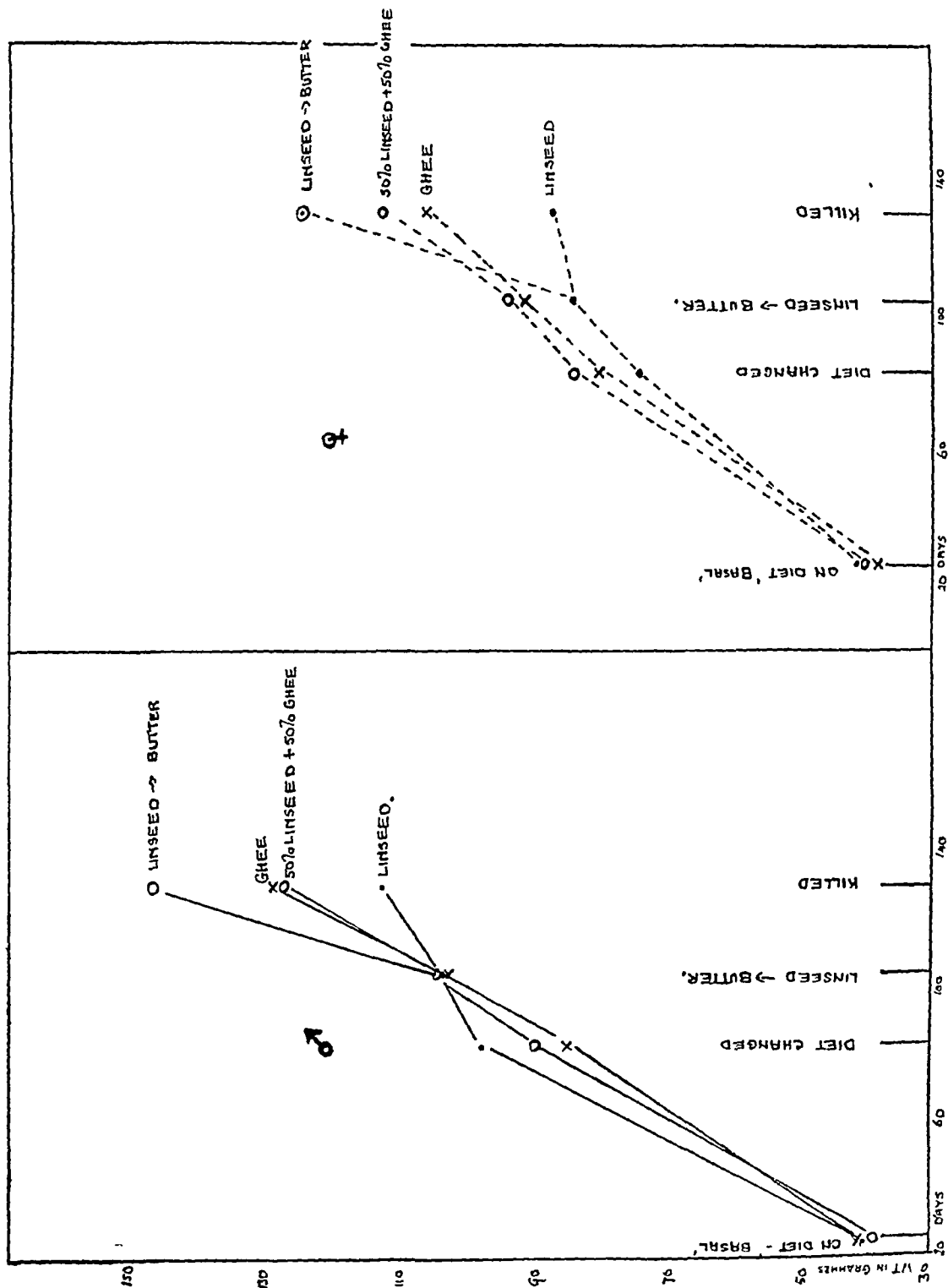
DISCUSSION

The results obtained in these series, though less dramatic because slower in onset than those obtained by feeding a synthetic-A diet, show that the vitamin A content of the ' Indian basal diet ' is markedly insufficient, and they bear out, from this particular aspect, the findings of McCarrison (1927), who tested the biological value of some of the Indian national diets

Series I —In the rats receiving the ' Indian basal diet ' typical symptoms of vitamin A deficiency occurred Xerophthalmia, stary coat, loss in weight

The rats receiving cabbage, either fresh or boiled, in addition to the basal ration exhibited none of these symptoms The partial destruction of vitamin A in the ' 4 hours' boiled cabbage ' group may be inferred from the fact that little or no vitamin A was found in the livers of the rats in this group They appear to have received sufficient vitamin A only for their daily needs and were therefore in the ' dangerous borderland of latent deficiency', in spite of their normal appearance and absence of other signs at post-mortem The experiment indicates that the Indian method of cooking vegetables for a considerable time in an open pan diminishes to a dangerous degree the vitamin A content of a diet already deficient in this respect

Series II —These experiments were repetitions of series I, using smaller amounts of cabbage In addition, another group received cabbage heated for 36 hours at 120°C in order to evaluate the influence of the mineral constitutents of cabbage in relation to the Indian basal diet The results were similar to those of series I , the addition of such small amounts of heated cabbage as 0 25 g fresh cabbage equivalent had only a small effect

Series III —Conflicting reports have been made regarding the assay of the vitamin A content of ghee (Bacharach, *loc cit* , Brahmachari, *loc cit*), chiefly because of the varying methods of its preparation and its adulteration with non-vitamin containing fats

Pure buffalo ghee imported from India was used in this series of experiments (A very brief summary will be given since the results from such a small number of rats as were available for the experiment do not justify definite statements, although they are indicative of certain conclusions)

The replacement of 2 per cent linseed oil by 2 per cent ghee and 4 per cent linseed oil by 4 per cent ghee in the basal diet appeared to make some difference to growth, whereas butter undoubtedly accelerated growth

Colorimetric estimation* of the vitamin A content (Carr and Price, *loc cit*) of the butter and ghee used, yielded the following results —

	Blue units	Yellow units
Butter	2 1 per gramme	5 6 per gramme
Ghee	*Nil*	*Nil*

The vitamin A content of the butter was low as shown by the above figures and by the absence of any stored vitamin in the livers of these rats No xerophthalmia had developed when the experiment was terminated , the gneeral condition

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* Kindly carried out for the author elsewhere

of both the basal and the ghee groups was sub-normal and inferior to the butter groups

The ' Indian basal diet ' because of its vitamin A insufficiency brings about, as already mentioned, a slow and insidious onset of symptoms, as in the human, under similar conditions   For this reason, and also because it approximates to the diet of the people of India, it may be of use in evaluating vitamin containing food-stuffs in terms of human response rather than rat response

Wills (1930) in her nutritional studies of the so-called ' pernicious anæmia of pregnancy ' carried out some experiments on rats, using diets relatively deficient in vitamins A and C, these diets being in some respects comparable to the ' Indian basal diet '

It is suggested that the ' Indian basal diet ' may be used with qualitative and quantitative modifications for the investigation of other problems of nutrition A few experiments are, therefore, in progress in relation to the production of osteomalacia, by withdrawing Radiostol from the diet and supplementing it with vitamin A

SUMMARY

1   An ' Indian basal diet ' corresponding approximately to the ordinary diet of Indians has been described and has been used for the biological assay of vitamin A

2.   This diet has been shown to be deficient in vitamin A

3   An assay of certain vitamin containing foods has been made, using the above as the basal diet

4   Such a diet has advantages over the standard synthetic diets when assessing the prophylactic and therapeutic value of vitamin A containing foods of India

5   It is suggested that the ' Indian basal diet ' may be utilized when investigating other problems of human malnutrition

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BIOCHEMICAL INVESTIGATIONS ON DIFFERENT VARIETIES OF BENGAL RICE

Part III.

ENZYMIC DIGESTIBILITY OF RICE STARCH AND PROTEIN ACTION OF SALIVARY AND PANCREATIC AMYLASE AS WELL AS OF PEPSIN AND TRYPSIN

BY

KALI PADA BASU, D SC (Dac), PH D (Munich),

AND

SATIPRASAD MUKHERJEE, M SC

(*From the Biochemical Section, Chemical Laboratories, Dacca University*)

IN two previous communications Basu and Sarkar (1935a and {b) have reported on the chemical composition of different varieties of Bengal rice and also on the digestibility of the same varieties towards taka-diastase   In this paper the action of salivary and pancreatic amylase towards starch and of pepsin and activated trypsin towards proteins of the different varieties of rice has been studied   Details about the different varieties and the methods of preparation will be found in Part I of this paper

DIGESTIBILITY OF RICE STARCH.

The action of the salivary as well as the pancreatic amylase on the starch of the different varieties of rice (both Aus and Aman) subjected to different treatments and in presence of all its constituents, the difference in the digestibility of sun-dried and parboiled rice and also the effect of polishing the rice on the digestibility of starch have been studied

The saliva was collected by chewing pure wax and chewing gum and was preserved in a refrigerator at 2°C to 3°C after mixing it with a few drops of toluene   Its activity was tested from time to time and found to remain constant   The sample of the pancreatic amylase (trypsin, Messis Carnrick & Co , U S A) was also preserved in the refrigerator and similarly tested

It is well known that due to inhibition by reaction products (Luers and Wasmund, 1922) the reaction velocity of these enzymic reactions gradually falls off   Readings were, therefore, limited to the first 15 minutes

Animal amylases have a higher optimum pH (pH 6 4 to 6 8) than amylase of plant origin (pH 4 6 to 5 2)   The reactions in case of starch digestion were carried out at the optimum pH 6 48

TABLE I

Salivary digestion

Showing the variation in the digestibility of different AUS varieties

| Variety | Time in minutes | MG OF REDUCING SUGAR | | | | Mean for all different treatments for 15 minutes |
		Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished	
Surjamukhi	4	9 87	11 92	10 68	12 26	
	8	14 75	18 92	18 03	15 59	
	15	15 75	20 49	19 03	19 73	18 75
PXS (8)	4	12 36	13 80	13 00	13 97	
	8	17 69	18 84	19 14	18 04	
	15	18 92	21 37	21 96	22 92	21 29
Bhutmari	4	12 35	13 57	14 80	15 58	
	8	18 04	19 34	20 63	23 81	
	15	18 92	20 13	22 20	25 48	21 68
Jhanji	4	12 35	12 35	14 80	15 58	
	8	17 84	19 73	18 92	23 81	
	15	18 84	18 63	22 20	24 89	21 64
Kataktara	4	9 87	11 55	12 55	13 00	
	8	15 75	17 12	17 72	17 72	
	15	17 72	20 13	20 81	21 52	20 04
Charnock	4	10 68	11 55	12 08	13 00	
	8	14 80	17 12	17 20	17 72	
	15	19 03	20 13	20 39	20 39	19 98
Dhairal	4	9 67	11 92	11 92	12 96	
	8	15 30	18 92	18 92	19 34	
	15	16 78	20 49	20 49	21 26	19 75
Kanmari	4	10 63	12 18	12 18	13 00	
	8	15 75	18 92	18 92	19 14	
	15	16 57	20 13	20 39	21 52	19 67
Mean for 15 minutes		17 81	20 44	20 94	22 22	20 35

(5)

TABLE II

Salivary digestion

Showing the variation in the digestibility of different AMAN varieties

Variety	Time in minutes	MG OF REDUCING SUGAR				Mean for all different treatments for 15 minutes
		Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished	
Indrasail	4	12 46	11 56	13 85	14 44	
	8	17 36	18 54	19 96	21 88	
	15	20 43	22 97	23 40	25 25	23 01
Dudshar	4	13 36	14 44	13 36	14 75	
	8	20 08	21 52	20 08	23 13	
	15	24 17	24 62	25 82	26 45	25 26
Jhingasail	4	12 46	11 99	12 60	13 25	
	8	16 83	18 54	20 08	22 11	
	15	20 43	21 88	23 40	24 90	22 65
Latisail	4	11 56	12 27	12 27	13 25	
	8	17 36	20 08	22 70	22 70	
	15	20 80	22 87	21 84	25 35	23 45
Bhasamanik	4	12 46	13 25	12 46	14 44	
	8	17 08	17 20	20 08	20 80	
	15	20 00	20 43	23 40	24 50	22 08
Nagra	4	11 56	12 01	14 21	14 21	
	8	17 36	18 02	20 80	21 22	
	15	20 43	21 20	24 17	24 76	22 64
DXI (34)	4	11 69	12 01	12 01	13 25	
	8	18 41	19 35	23 09	22 06	
	15	20 43	21 63	24 04	23 97	22 51
Mean for 15 minutes		20 95	22 23	24 15	25 01	23 08

TABLE III

Pancreatic digestion

Showing the variation in the digestibility of different AUS varieties

| Variety | Time in minutes | Mg of reducing sugar | | | | Mean for all different treatments for 15 minutes |
		Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished	
Surjamukhi	4	12 53	13 03	12 53	13 53	
	8	19 05	19 55	18 04	19 55	
	15	21 06	22 06	20 05	22 05	21 3
PXS (8)	4	10 53	11 53	14 04	14 04	
	8	13 53	17 54	20 05	21 55	
	15	17 04	20 55	23 56	23 05	21 05
Bhutmari	4	13 03	13 53	10 53	11 03	
	8	17 54	18 05	16 03	18 04	
	15	20 05	21 05	19 54	21 05	20 42
Jhanji	4	12 53	12 03	11 03	11 53	
	8	18 05	16 04	16 79	15 54	
	15	19 55	20 55	18 04	20 04	19 54
Kataktara	4	14 04	14 54	13 53	14 54	
	8	18 05	20 55	19 79	20 55	
	15	20 05	21 05	21 55	23 05	21 49
Charnock	4	11 70	12 93	12 00	12 31	
	8	16 31	17 85	17 93	18 24	
	15	17 54	19 08	21 39	21 70	19 92
Dhairal	4	14 16	14 77	11 70	12 93	
	8	18 47	18 47	18 62	18 24	
	15	19 08	19 70	20 47	21 70	20 23
Kumari	4	13 85	14 46	13 08	13 54	
	8	18 77	19 08	17 39	18 47	
	15	21 85	22 16	18 62	22 93	21 39
Mean for 15 minutes		19 53	20 77	20 4	21 95	20 66

TABLE IV

Pancreatic digestion

Showing the variation in the digestibility of different AMAN varieties

Variety	Time in minutes	MG OF REDUCING SUGAR				Mean for all different treatments for 15 minutes
		Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished	
Indrasail	4	11 53	12 53	12 02	12 03	
	8	15 29	16 04	18 29	18 09	
	15	19 05	18 04	20 54	20 85	19 62
Dudshar	4	12 03	12 53	14 04	14 54	
	8	16 04	17 04	18 55	20 05	
	15	18 55	19 30	21 05	21 05	19 99
Jhingasail	4	13 54	14 16	12 08	14 16	
	8	17 85	18 47	17 62	18 08	
	15	19 08	20 31	19 35	20 05	19 69
Latisail	4	12 31	13 54	13 31	14 77	
	8	16 85	17 85	17 00	19 08	
	15	17 85	19 08	18 74	20 93	19 15
Bhasamanik	4	11 03	12 03	12 53	12 03	
	8	15 79	16 54	16 79	17 04	
	15	18 04	19 05	20 04	20 05	19 29
Nagra	4	12 62	13 54	13 00	13 54	
	8	15 39	16 62	17 62	17 24	
	15	19 08	20 01	19 35	20 93	19 84
DXI (34)	4	11 08	12 31	12 39	12 31	
	8	14 16	16 70	17 39	16 62	
	15	17 54	18 77	19 47	19 08	18·71
Mean for 15 minutes		18 46	19 22	19 58	20 33	19 39

Experimental

The various samples of rice were powdered and sieved through a 60-mesh sieve and the requisite quantity was taken and treated with a little cold water and boiled for 8 to 10 minutes It was then cooled and made up to known volume so that about 1 25 per cent suspension of starch was obtained A little toluene was also added to prevent bacterial action The substrate was prepared every day before the experiment

Each reaction mixture was made up as follows , 25 c c of starch suspension, 10 c c of M/5 phosphate buffer having a pH 6 48, 1 c c of 0 2 N NaCl (which behaves as an activator of both the salivary and pancreatic amylases) and 3 c c of H_2O were taken in a flask and whole kept in a constant temperature bath at 37°C $\pm$ 0 1 When the contents of the flask acquired the temperature of the bath, 2 c c of saliva in case of salivary digestion was added, thus making the volume equal to 41 c c In case of hydrolysis achieved by the pancreatic amylase, 5 c c of 0 8 per cent trypsin instead of 2 c c of saliva and 3 c c of H_2O, was added to the reaction mixture, thus making the final volume equal to 41 c c Five c c of the reaction mixture was withdrawn every 4, 8 and 15 minutes and the reaction was stopped by adding a few drops (10 to 12) of N HCl The reducing sugar formed, in both cases, was estimated by the method of Willstratter, Waldschmidt-Leitz and Hesse (1923)

Each time a reading was taken immediately after adding the enzyme to the reaction mixture This gave the reading at zero minute Duplicate estimations were carried out in every case and the mean result is given in the above tables

DIGESTIBILITY OF RICE PROTEIN

The action of the proteolytic enzymes, pepsin (Merck) and activated trypsin (Messrs Carnrick & Co , U S A) on the proteins of the different varieties of rice subjected to different treatments have been studied Here also the enzymes were preserved in a refrigerator

Experimental

The extent of hydrolysis was measured in a 25 c c measuring flask Twenty c c of 4 per cent rice powder in water was brought up to the desired pH (e g , pH 2 in case of peptic digestion) with the addition of dil HCl or NaOH in drops Clark and Lube's series of indicators for the determination of pH were used for this purpose The flask was then kept in a thermostat at temperature of 40°C In the case of digestion by the proteolytic enzyme pepsin, 0 04 g of pepsin (Merck) was added to each of the measuring flasks under investigation and the mixture diluted to 25 c c by adding water previously heated to 40°C Five c c. was withdrawn from time to time and added each time to 50 c c 95 per cent alcohol to stop the reaction and then titrated with standard alcoholic KOH (N/25) by means of a micro burette, using thymolphthalein (0 5 per cent) as the indicator till a permanent blue colour was obtained (Willstratter and Waldschmidt-Leitz, 1921) Each time a blank experiment was made just adding the enzyme to the substrate to give the zero reading

In case of tryptic digestion 0 04 g of trypsin (obtained from Messrs Carnrick & Co , U S A , and known by the name of ' Trypsogen ') was put into one 25 c c measuring flask and 2 c c of phosphate buffer (M/15) at pH 8 5 and 2 c c of 0 005 M cystine solution at pH 8 5 was added to activate the enzyme (*vide* Grassmann, 1930)   The flask was then kept for half an hour in the thermostat at 40°C for the purpose of activating the trypsin by the cystine solution   After the activation of the enzyme, 20 c c of the substrate at pH 8 5 was added to it and the reaction mixture diluted to 25 c c by adding water   The determination of the tryptic hydrolysis is just the same as in peptic digestion   In this case also a blank experiment was carried out and the experimental result is given in the following tables —

TABLE V

Showing the variation in the digestibility of different AUS varieties by pepsin at pH 2 and temperature 40°C

Variety		Time in hours	AMOUNT OF ALCOHOLIC KOH (N/25) REQUIRED IN C C			
			Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished
Surjamukhi	{	1	0 354	0 398	0 442	0 484
		3	0 524	0 619	0 750	0 749
PXS (8)	{	1	0 400	0 556	0 580	0 629
		3	0 677	0 629	0 774	0 810
Bhutmari	{	1	0 599	0 649	0 896	0 999
		3	0 799	0 896	1 197	1 197
Jhanji .	{	1	0 599	0 746	0 792	0 652
		3	0 798	0 885	0 980	0 839
Kataktara	{	1	0 375	0 375	0 435	0 532
		3	0 606	0 466	0 559	0 629
Charnock	{	1	0 388	0 406	0 679	0 609
		3	0 582	0 723	0 970	1 100
Dharial	{	1	0 582	0 582	0 485	0 603
		3	0 776	0 801	0 727	0 778
Kumari	{	1	0 291	0 385	0 582	0 533
		3	0 485	0 623	0 727	0 827
Mean for 3 hours			0 662	0 705	0 835	0 870

TABLE VI

Showing the variation in the digestibility of different AMAN varieties by pepsin at pH 2 and temperature 40°C

Variety	Time in hours	Amount of alcoholic KOH (N/25) required in c c			
		Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished
Indrasail	1	0 600	0 639	0 630	0 810
	3	0 720	0 810	0 970	1 016
Dudshar	1	0 530	0 623	0 796	0 796
	3	0 720	0 827	0 936	0 982
Jhingasail	1	0 970	0 970	1 021	1 010
	3	1 261	1 122	1 353	1 402
Latisail	1	0 291	0 323	0 582	0 533
	3	0 485	0 723	0 827	0 927
Bhasamanik	1	0 374	0 400	0 468	0 468
	3	0 561	0 623	0 741	0 810
Nagra 68/6	1	0 981	1 021	1 215	1 215
	3	1 215	1 303	1 402	1 402
DX 9 (34)	1	0 582	0 473	0 485	0 468
	3	0 776	0 623	0 776	0 970
Mean for 3 hours		0 819	0 862	1 001	1 071

TABLE VII

Showing the variation in the digestibility of different AUS varieties by trypsin at pH 8 5 and temperature 40°C

Variety	Time in hours	AMOUNT OF ALCOHOLIC KOH (N/25) REQUIRED IN C C			
		Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished
Surjamukhi	1	0 301	0 354	0 531	0 677
	3	0 575	0 619	0 885	0 822
PXS (8)	1	0 481	0 530	0 977	0 951
	3	0 723	0 822	1 257	1 257
Bhutmari	1	0 399	0 599	0 574	0 849
	3	0 599	0 749	0 848	0 971
Jhanji	1	0 399	0 466	0 233	0 466
	3	0 648	0 699	0 666	0 746
Kataktara	1	0 375	0 512	0 559	0 609
	3	0 612	0 769	0 725	0 725
Charnock	1	0 679	0 713	0 970	1 012
	3	0 917	1 071	1 164	1 210
Dhairal	1	0 776	0 706	0 776	0 812
	3	0 970	1 067	1 067	1 210
Kumari	1	0 388	0 388	0 582	0 623
	3	0 582	0 623	0 970	0 970
Mean for 3 hours		0 704	0 802	0 95	1 001

TABLE VIII

Showing the variation in the digestibility of different AMAN varieties by trypsin at pH 8 5 and temperature 40°C

Variety	Time in hours	Amount of alcoholic KOH (N/25) required in c c			
		Sun dried non polished	Sun dried polished	Parboiled non polished	Parboiled polished
Indrasail	1	0 810	0 990	1 064	1 064
	3	0 990	1 215	1 354	1 451
Dudshar	1	0 990	0 970	1 029	0 982
	3	1 080	1 124	1 169	1 215
Jhingasail	1	1 358	1 215	1 456	1 303
	3	1 649	1 746	1 746	1 701
Latisail	1	0 388	0 485	0 582	0 603
	3	0 582	0 726	0 970	1 215
Bhasamanik	1	0 167	0 489	0 468	0 533
	3	0 654	0 685	0 761	0 970
Nagra 68/6	1	1 122	1 215	1 300	1 215
	3	1 496	1 649	1 729	1 911
DX 9 (34)	1	0 873	0 970	0 873	1 029
	3	1 164	1 215	1 261	1 203
Mean for 3 hours		1 088	1 190	1 290	1 519

DISCUSSION

It will be evident from the above tables that both amongst the Aus and the Aman rice the individual varieties show variations from one another and also from the average both in the case of amylolytic as well as of proteolytic degradation Judging the result as a whole it will be seen that both the starch and protein of the

Aman rice are more digestible than those of the Aus   Only towards the pancreatic amylase the Aus starch is slightly more digestible than the Aman starch   Similar results have also been observed by Basu and Sarkar (1935a and b) working with taka-diastase   It will also be seen that salivary amylase hydrolyses rice starch more quickly than the taka-diastase

This investigation supports the popular belief that on the whole Aman varieties are more readily digested than the Aus ones

Tables V to VIII also make it clear that the proteins of rice are more readily hydrolysed by activated trypsin than by pepsin   This fact might be borne in mind when prescribing this enzyme preparation to patients

Parboiling increases the rate of digestion of rice starch and also of rice protein both in Aus and Aman varieties   This is to be expected in view of the fact that in the process of parboiling, rice starch is probably dextrinized to some extent and in the case of protein digestion this is most probably due to some hydrolysis of the rice proteins

In case of both Aus and Aman, polished rice starch is more digestible than that from the corresponding non-polished rice   The bran thus appears to contain some inhibitors for the action of the enzymes studied   Polishing has very little effect on the digestibility of rice proteins   It should be noted that the non-polished Bhutmari and Jhanji, which are highly coloured, undergo very little hydrolysis by trypsin, whereas their hydrolysis by pepsin is not in the least retarded   So it is suggestive that the deep-red coloured coating of these two varieties contains some inhibitor for the action of trypsin but not for pepsin

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BIOLOGICAL VALUE OF THE PROTEINS OF GREEN GRAM (*PHASEOLUS MUNGO*) AND LENTIL (*LENS ESCULENTA*).

Part I.

BY THE BALANCE SHEET METHOD

BY

KALI PADA BASU, D sc (Dac), PH D (Munich),

MADHAB CHANDRA NATH, M sc ,
Lady Tata Memorial Scholar,

AND

MUHAMMAD OSMAN GHANI, M sc.
(*From the Biochemical Section, Chemical Laboratories, Dacca University*)

PROTEIN is one of the essential ingredients of food-stuffs and is indispensable for the formation of body tissues   But proteins from all sources are not equally utilized by the body   Those lacking in essential amino-acids like cystine, histidine, proline, tryptophane etc , are simply de-aminized in the system, the nitrogen being excreted in the form of urea and the residue utilized like non-nitrogenous substances in the body   The percentage of digested protein that is utilized for the maintenance of the nitrogenous integrity of the tissues and for tissue-formation is known as the biological value of the protein   It is evident that a determination of this biological value of proteins of our food-stuffs is essential before a suitable diet can be recommended

Pulses contain a high percentage of protein, in some cases almost as high as that of meat, and form the chief source of proteins in the diets of the Indians, especially of the vegetarians   The important problem of the determination of the biological value of the proteins of the two common pulses, green gram and lentil, forms the subject-matter of this investigation

(789)

The pulses *Phaseolus mungo* and *Lens esculenta* are very widely cultivated in almost all parts of India and their consumption is also very great   The former forms one of the ingredients of daily diet in west Bengal and the east Bengal people take the latter variety of pulse almost daily

The vernacular names of *Phaseolus mungo* are as follows —

Sanskrit, Mudgas , Bengali, Mug , Hindi, Harimung , Telegu, Peshu , Guzrati, Maghila , Persian, Bunu mash , Arabic, Mag , English, Green gram

The pulse *Lens esculenta* has the following names in different languages —

Sanskrit, Masura , Bengali, Masuri, Masurika and Mangalyaka , Hindi, Masur , Tamil, Misur , Telegu, Cirisonmalu , Persian, Bunu surkha , Arabic, Adam , English, Lentil , Latin, Erveylens

Susrut, one of the ancient thinkers of India, placed both the pulses at the top of all the rest, saying —

ऋते मूद्ग मसुराभ्या मन्ये त्वाध्यान कारका ।

which means that all the pulses save and except green gram and lentil, cause formation of wind within the system when taken internally

We find in *Smriti*, one of the valuable works of ancient India, that *Phaseolus mungo* has been prescribed for those who have just received a mental shock due to the loss of their parents (husbands in case of women)

The verse says —

हैमन्तिक सितास्विन्न धान्य मूद्ग स्तिलायवा. ।

The superiority of *Phaseolus mungo* over all the other pulses can also be supported from a verse in *Sruti* —

मूद्ग"र्प्सृहोति ।

Indian vegetarians hesitate to take *Lens esculenta* and the orthodox Hindus and Hindu widows think it rather sinful on their part to use it along with their diet

It was thought desirable, therefore to have a thorough and complete investigation of the proteins of the two pulses (both biological and chemical) in order to gain some insight into their nutritive values and to find out whether the proteins from these sources can confidently be recommended from the scientific point of view as suitable substitutes for costly proteins of animal origin

Investigations of the biological properties of isolated proteins and definite protein mixtures have been pursued for many years in a number of ways   Thomas (1909) proposed the term biological value of a protein to express the percentage of absorbed nitrogen which was retained by the body for the repair and the construction of nitrogenous tissues and hence differences in digestibility of different proteins did not affect their biological value   The term thus expressed the relative value of different proteins for compensating the daily nitrogenous loss in the body Thomas carried out a series of heroic experiments on himself   He assigned to the proteins of milk, meat and fish biological values approximately 100, indicating complete utilization of the absorbed nitrogen   To the proteins of corn, wheat, rice, potatoes and peas, the values 30, 40, 88, 79 and 56 respectively were assigned

by him   A study of the details of the experiments of Thomas detracts greatly
from their significance   There were no preliminary or transition periods before
the actual experiments were taken up and no attempt was made to insure a constant
intake of nitrogen and calories during the short periods of investigation and the
different proteins were fed at different levels of intake

Sherman (1920) and Sherman and Winters (1918) have carried out metabolism
experiments on human subjects and maintain that cereal proteins with minimal
additions of milk proteins seem to function with very great efficiency in the main-
tenance of nitrogen equilibrium

Martin and Robinson (1922) adopted the procedure of Thomas for the
determination of the biological value of some important food-stuffs, though they
criticized his method from certain standpoints

McCollum *et al* (1911, 1914) applied this method on pigs and Wagner (1923)
on children   Mitchel (1924*a* and *b*) has elaborated a balance sheet method of
biological evaluation of proteins using albino rats and has thoroughly discussed
the various sources of error and the degree of accuracy obtained therein   This
modified method consists in a determination of the average daily nitrogenous
balance sheet on a diet containing a definite percentage of the protein under
investigation   Employing this method, Mitchel (1924*b*) and Mitchel *et al* (1924,
1926 1927) have determined the biological value of the proteins of a large
number of cereals and other articles of food   The method of Mitchel with slight
modifications is now used in almost all laboratories and has also been adopted
by us in this investigation

CHEMICAL COMPOSITION

The air-dried pulses were decorticated and the seeds after removal of seed-
coats, were ground to a fine powder and allowed to pass through a sieve of sixty
mesh   Moisture was determined by drying the specimens of flours at 105°C for
10 hours and the percentage composition of other constituents was determined
according to the method recommended by the Association of Official Agricultural
Chemists   The following is the result found on analysis   (The sieved powder
of the pulses was used in the experiments on the determination of biological
value) —

TABLE I

Analysis of pulses used as sources of protein

Pulse	Moisture (per cent)	Total nitrogen (per cent)	Protein N × 6 25 (per cent)	Ether extrac tives (per cent)	Ash (per cent)	Crude fibre (per cent)	Carbohydrate (by difference)
Phaseolus mungo	15 00	3 72	23 26	2 88	3 39	0 81	50 94
Lens esculenta	15 11	3 62	22 60	2 09	1 78	0 86	53 92

<div align="center">TECHNIQUE</div>

In the determination of biological value various devices have been adopted as regards the construction of metabolism cages for the quantitative separation of the urine and the fæces of the experimental animals   In the construction of such cages careful arrangements must be made so that there may not be any contamination of the fæces with the urine as well as with the experimental diet   The animals must also have no access to the fæces thus avoiding consumption of the fæces   The metabolism cages used in these experiments were those described by Akroyd and Hopkins (1916), with the detachable wire-floor introduced by Boas (1924), and with glass separators introduced by Pain of the Cancer Hospital Research Institute   The cages and separators were made in the local market according to directions   Such cages have also been used by Boas Fixsen (1930, 1932) in her experiments for the determination of biological values of wheat, maize and casein (*cf* also Chick and Roscoe, 1930)   .

Two adult male rats (weight about 250 g) were placed in each cage   The pair was treated as a single unit

The determination of the fæcal and urinary nitrogen on a nitrogen-free diet was first undertaken, and later on similar determinations on diets containing the different percentages of proteins (5 per cent, 10 per cent, 15 per cent) of the pulses were carried out   In experiments with the nitrogen-free ration, the rats were kept for a preliminary period of three days during which no collection was made and the collection of the fæces and urine were made for the subsequent four days   With the protein diets there was a pre-experimental period of four days and the experiment proper lasted for a further period of four days

The experimental diets were prepared by adding the necessary ingredients in the desired proportion as shown in Table II   The diets so prepared include the essential inorganic ions and the available sources of energy in a properly balanced proportion   This composition was adopted by Boas Fixsen, Chick and Roscoe and later by other workers in the field and has been found to conform, more or less, to the requirements of the animal body so far as the non-protein nutritive constituents of the diet are concerned   The constituents of the diet were thoroughly mixed up to make it as uniform as possible   About 600 g of the diet was prepared at a time and put in a wide-necked stoppered bottle and kept in the refrigerator when not in use

Samples were then taken out and protein content determined by estimating the nitrogen by the Kjeldahl method   The approximate amount of the diet required every day by each set of rats was weighed into the daily feeding cups, cooked in water until the odour of the raw flour disappeared and made into a thick paste to prevent scattering   Although the rats showed no aversion to the uncooked diet the water-cooked one was very well taken   The provision of adequate amounts of the vitamin B complex in the experimental diet is essential for the maintenance of appetite and hence for a successful determination of the biological value   Although the pulses contain some amount of the vitamin B complex the amount was further supplemented by the addition of 2 c c of a dilute solution of yeast preparation to the cooked paste   The solution contained 0 002 g nitrogen per c c   Hence the amount of protein from this source was negligible   The cups were then placed in the chambers at either ends of the two arms of the metabolism

cage In weighing out the daily food care was taken so that the food might not be much greater than the average daily consumption thus avoiding a large residue Every 24 hours the traces of residual food remaining in the cups were collected and preserved in the refrigerator At the end of the experiment (four days) all of the residual diet was dried in an oven and made into a uniform powder Nitrogen was estimated either in aliquot or of the whole The amount of nitrogen contained in the diet given in the cups during the whole experiments was calculated and by subtracting from this the nitrogen remaining with the uneaten residue the total nitrogen intake was obtained The amount of food consumed was also calculated from this

To prevent decomposition of urine and consequent loss of ammonia 5 c c of a 5 per cent solution of carbolic acid and 1 c c of a 10 per cent solution of thymol were placed daily in the small conical flask used to collect the urine

The fæces were taken out daily from the collecting beaker and preserved in the refrigerator If soiled with urine the fæces were previously washed and the washings added to the urine bottle At the close of the experiment all of the fæces were dried in an oven, made into a uniform powder in a mortar and an aliquot portion weighed out for nitrogen determination

The metabolism cage, funnel, beaker and separator placed for the collection of fæces and urine were daily washed with dil acetic acid and distilled water The urine and washings were filtered through a thin cloth into a 500 c c measuring flask The flask was kept in the refrigerator At the end of the experiment the urine was diluted to the mark with water and nitrogen determined in 25 c c of the solution

By dividing the total urinary nitrogen the total fæcal nitrogen and the total nitrogen intake by 8—the corresponding figures per rat per day were obtained The rats were weighed at the beginning and close of the experiment There was at least one week interval between two consecutive experiments with the same set of rats

The experiments with the nitrogen-free ration were similarly conducted, there being a pre-experimental period of three days during which no collection was made The metabolic nitrogen in the fæces, the urinary nitrogen of endogenous origin and the amount of food intake per rat per day were calculated out similarly The metabolic nitrogen per g of food consumed was also calculated for each set of rats and the results applied in finding out the metabolic nitrogen of the protein diet feeding period After about three months these experiments were repeated

An essential condition for the success of the method is that dietary proteins should not be utilized as a source of energy and hence the results were accepted only of those experiments in which the food intake and hence the intake of non-protein calories were sufficient (not less than 120 kg calculated per day per kg body-weight)

Materials used .—

Table I gives the analysis of pulses used as sources of protein

Table II gives the composition of diets used in these experiments

TABLE II

Composition of diet

Diets	Nitrogen free	Phaseolus mungo			Lens esculenta			Mixed diet
		(grammes)	(grammes)	(grammes)	(grammes)	(grammes)	(grammes)	(grammes)
Percentage of protein approximate		15 (per cent)	10 (per cent)	5.8 (per cent)	15 (per cent)	10 (per cent)	5.6 (per cent)	10 (per cent)
Green gram	0	395	263	150				132
Lentil	0				400	264	150	132
Chopped sugar	90	54	42	54	42	45	54	45
Hardened arachis oil	130	72	72	72	72	72	72	72
Cod liver oil	20	12	12	12	12	12	12	12
Salt mixture	50	24	24	24	24	24	24	24
Calcium carbonate	8	6	6	6	6	6	6	6
Corn starch	735	42	181	285	50	175	292	180
Percentage of protein in the diet (N×6.25)	0.104 (per cent)	15.2 (per cent)	(i) 10.2 (per cent) (ii) 11.6 (per cent)	5.8 (per cent)	15.1 (per cent)	(1) 9.85 (per cent) (2) 11.3 (per cent)	5.62 (per cent)	10.02 (per cent)
Total calorie intake value per g of diet	5	3.93	4.13	4.37	4.01	4.13	4.38	4.14

CALCULATION OF THE BIOLOGICAL VALUE

Martin and Robison, and Mitchel have fully discussed the various problems involved in the calculation of the biological value of proteins from the data obtained from the nitrogen balance experiments

The formula used by Mitchel, and which has also been adopted by us in this investigation, is as follows —

$$\text{Biological value} = \frac{\text{Body nitrogen retained}}{\text{Food nitrogen absorbed}} \times 100 \tag{1}$$

this can be written as

$$100 \times \frac{\text{True nitrogen intake} - \text{True urinary nitrogen output}}{\text{True nitrogen intake}} \tag{2}$$

$$= 100 \times \left[1 - \frac{(\text{Total urine N} - \text{Endogenous urine N})}{\text{Total nitrogen intake} - (\text{Total fæcal N} - \text{Metabolic N})} \right] \tag{3}$$

Martin and Robison, on the other hand, used a slight modified method of calculation They plotted the nitrogen intake as abscissa against nitrogen output as ordinate and showed that biological value $= 100 (1-\tan \phi)$, where ϕ is the slope of the straight line obtained by joining the plotted point to the point corresponding to the nitrogen output on a nitrogen-free diet These two methods of calculation are practically identical

Boas Fixsen (*loc cit*), and Boas Fixsen and Jackson (*loc cit*), adopted the latter method of calculation To express the output they introduced the term ' true output ' which is made up of the urinary nitrogen plus the endogenous fæcal nitrogen They are hardly justified in adding the metabolic nitrogen to the urinary nitrogen to obtain the nitrogen output But this affects the biological value only very slightly

METABOLIC NITROGEN IN THE FÆCES

One factor which is essential for the calculation of biological value of proteins and about which there is still some uncertainty in spite of various controversies on the subject, is the metabolic nitrogen of the fæces (output of nitrogen in the fæces on a nitrogen-free diet) Thomas (*loc cit*) maintained that metabolic nitrogen was independent of food intake Martin and Robison (*loc cit*), on the other hand, thought that the composition of the diet, in particular its content of undigestible materials, might affect the excretion of metabolic nitrogen in the fæces Boas Fixsen and Jackson (*loc cit*), as a result of some experiments on adult rats, have come to the conclusion that metabolic nitrogen is independent of food intake

Mitchel (1924a), working with younger rats, has shown that metabolic nitrogen in the fæces on a protein containing diet is related to the amount of food consumed and may be measured from the total excretion of fæcal nitrogen on a nitrogen-free diet The latter, according to Mitchel may be used with the most confidence when the roughage content of the nitrogen-free diet approximates that of the protein containing diet

Recently the whole question has been thoroughly discussed by Schneider (1934), who makes use of nitrogen metabolism data on nitrogen-free diet obtained as the result of 1,160 determinations Schneider has pointed out the sources of

error in the experiments of Boas Fixsen and Jackson and has definitely shown that metabolic nitrogen of fæces does vary with the food intake although the variation is not as pronounced in the case of adult rats as in the case of growing rats   The observations and discussions of Schneider definitely show that metabolic nitrogen of a rat is made up of two parts—one part is dependent only on the weight of the rat and does not vary with the food intake   This constant part, which, we suggest, be called ' nitrogen of the fasting metabolism ', is the intercept on the ordinate of the straight line obtained by plotting grammes of food matter consumed per day as abscissa against   mg   of metabolic nitrogen per day as ordinate   The other part of the metabolic nitrogen varies with food intake   We have calculated the metabolic nitrogen both on the basis of Schneider's principle and also as being directly proportional to the food intake   The results are applied in the calculation of biological value   The resulting variation in the result obtained is practically negligible

In using Schneider's principle, nine points are plotted (Fig 1) using the data from the nitrogen-free experiments   These points lie grouped about a straight line which cuts an intercept of 7 3 from the ordinate   This is taken to be the nitrogen' of the fasting metabolism of the animals of the size used in our experiments   Similarly, grammes of food matter consumed are plotted against mg of metabolic nitrogen per gramme of food consumed and the curve is obtained (*see* Fig 2)   At intakes higher than 15 grammes, the curve becomes practically parallel to the abscissa at a distance of 1 6

The metabolic nitrogen in the protein feeding experiments is obtained from Fig 1 from a knowledge of the total food consumed   For intakes of food greater than 15 grammes, the metabolic nitrogen is obtained from Fig 2, i e , for intakes greater than 15 grammes 1 6 is taken to be the ratio of metabolic fæcal nitrogen to the intake of food

However, this method of finding out the metabolic nitrogen from the graph does not take into account the idiosyncrasy of the individual rats   Calculation by this method would have been most accurate if a large number of determinations could have been made with every rat, consuming varying amounts of food in each determination

The Endogenous Nitrogen of Urinary Origin

The excretion of nitrogen in the urine resulting from the catabolism of the tissues is not markedly depressed, if at all, by protein feeding   There seems to be a true basal catabolism of nitrogenous substances in the tissues, such that the amount of nitrogen of body origin excreted in the urine, when an animal is on a protein containing dietary, may be satisfactorily measured by the total excretion of nitrogen in the urine in an adjacent period of feeding on a nitrogen-free diet   This endogenous catabolism of nitrogen does not seem to be affected by an influx of amino-acids from the intestinal tract

The results of experiments with the nitrogen-free ration are given in Table III

The results of metabolism experiments with the proteins of green gram and lentil are shown in Tables IV and V respectively

FIGURE 1

Food consumed (g.).

Figure 2

Metabolic nitrogen per gm. of food (mg.).
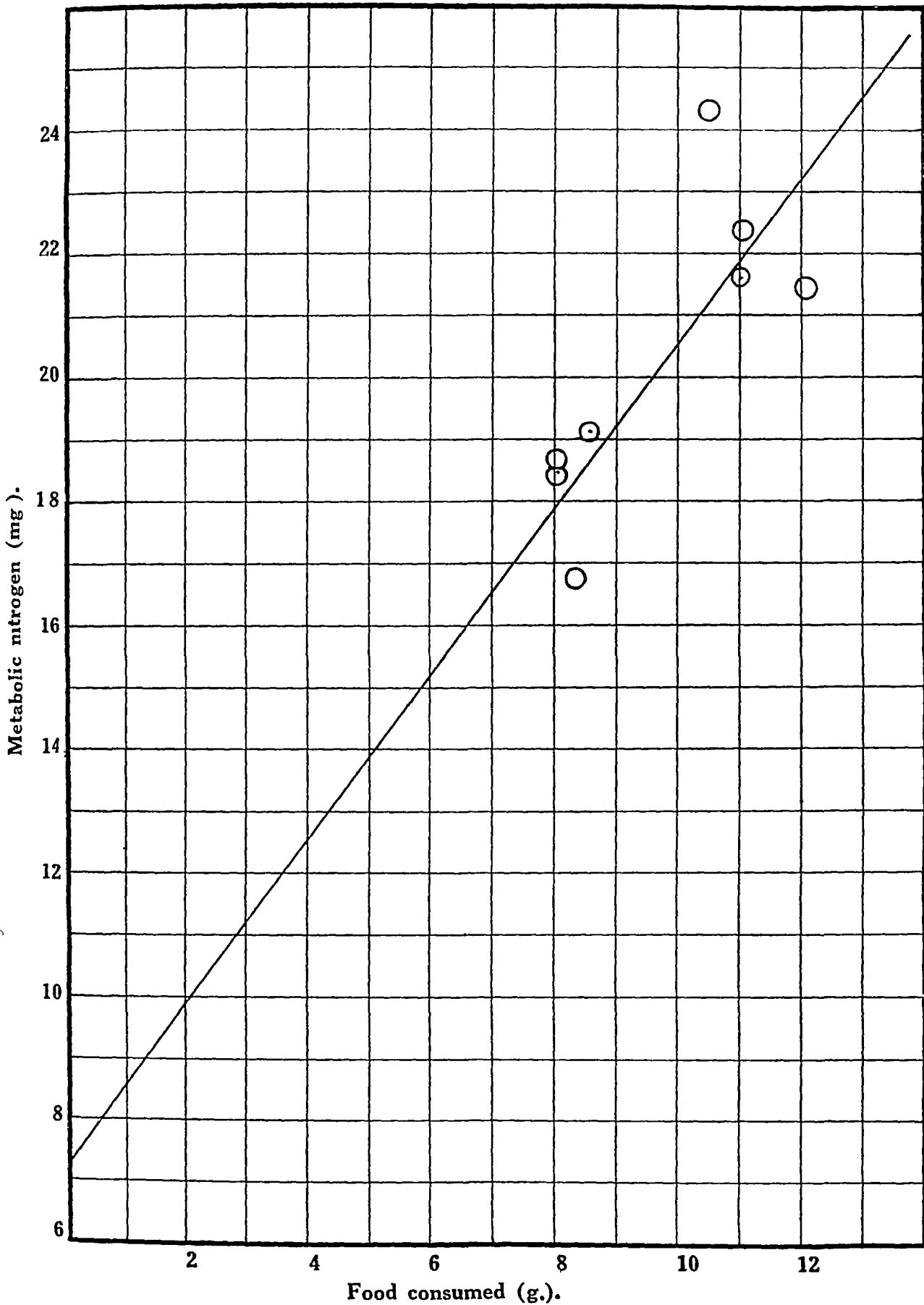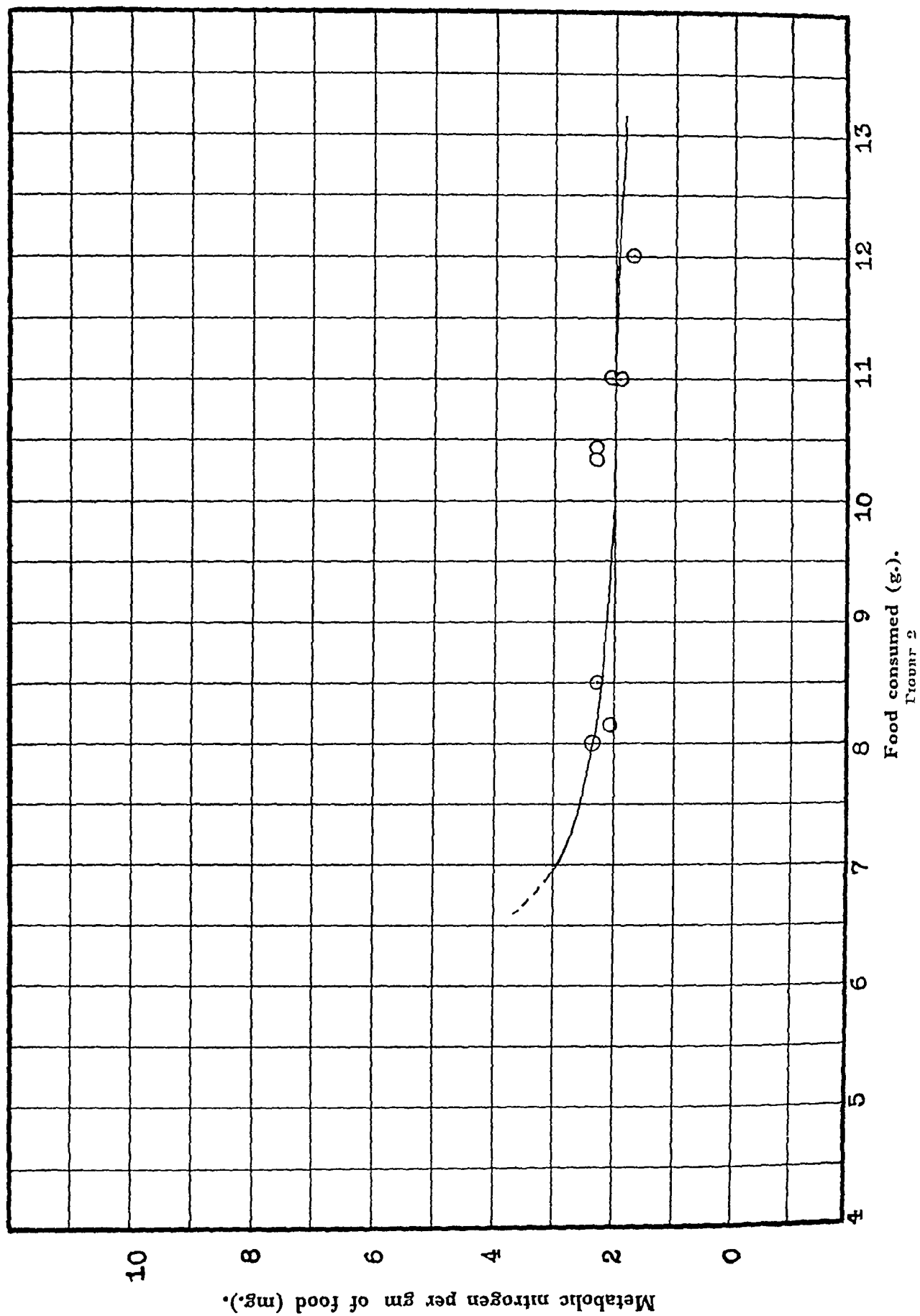
TABLE III.

Experiments with nitrogen-free ration

Date	Experiment number	Rat numbers	Average body-weight	Average change in body weight	Food intake (g)	Urine N₂ (mg)	Metabolic N₂ (mg)	Metabolic N₂ per g of food (mg)	Remarks
29-9-34	(I)	13 and 14	221 2	0 3	8 5	42 3	19 1	2 247	Used in the calculation of the biological value of green gram
29-9-34	(II)	12 and 15	264 8	−4 3	8 0	54 0	18 6	2 325	
29-9-34	(III)	10 and 11	255 0	1 5	12 0	56 4	20 4	1 700	
12-1-35	(I)	13 and 14	235 5	−1 5	10 4	41 8	24 3	2 336	Ditto of lentil
12-1-35	(II)	12 and 15	267 0	−2 0	11 0	55 1	22 3	2 027	
12-1-35	(III)	10 and 11	259 5	−1 7	11 0	55 9	21 6	1 964	
17-5-35	(I)	21 and 22	225 5	−2 0	7 2	24 1	12 7	1 764	Ditto of the mixed diet
17-5-35	(II)	23 and 25	283 5	−2 0	8 0	24 8	18 5	2 312	
17-5-35	(III)	29 and 30	273 5	−2 7	8 2	21 7	16 8	2 050	

TABLE IV

Biological value of Phaseolus mungo

Protein content (per cent)	Experiment number	Rat numbers	Average body weight (g)	Average change in body-weight (g)	Food intake (g)	Non protein calculated per kg of body-weight	N₂ Intake (mg) Total	N₂ Intake (mg) True	Urine N₂ (mg) Total	Urine N₂ (mg) Endogenous	Urine N₂ (mg) True	Faecal N₂ (mg) Total	Faecal N₂ (mg) Endogenous	Faecal N₂ (mg) Exogenous	Biological value	Mean
5.8	(I)	13 and 14	226.5	−0.5	14.9	273	139.6	130.6	90.8	42.3	48.5	42.5	33.5	9.0	63	63
5.8	(II)	12 and 15	263.2	4.8	17.6	276	165.1	157.3	108.4	54.0	54.4	48.6	40.8	7.8	65	
5.8	(III)	10 and 11	246.2	3.5	18.0	304	167.1	148.7	112.8	56.4	56.4	49.0	30.6	18.4	62	
11.6	(IV)	13 and 14	219.5	−0.5	7.1	119	131.8	111.9	99.3	42.3	57.0	35.8	15.9	19.9	49	52
11.6	(V)	12 and 15	260.2	−1.7	8.6	123	159.9	137.4	116.1	54.0	62.1	42.5	20.0	22.5	55	
11.6	(VI)	10 and 11	251.5	0.5	10.3	152	192.7	166.7	136.5	56.4	80.1	43.5	17.5	26.0	52	
15.0	(VII)	13 and 14	228.5	3.2	12.3	179	307.1	285.5	197.2	42.3	154.9	49.1	27.5	21.6	46	45
15.0	(VIII)	12 and 15	267.0	9.7	15.5	192	387.2	362	246.7	54.0	192.7	61.2	36.0	25.2	47	
15.0	(IX)	10 and 11	253.0	10.5	14.9	197	371.2	340.7	251.7	56.4	195.3	55.8	25.3	30.5	43	

TABLE V

Biological value of Lens esculenta

Protein content (per cent)	Experiment number	Rat numbers	Average body weight (g)	Average change in body weight (g)	Food intake (g)	Non protein calculated per kg of body weight	N₂ INTAKE (MG) Total	N₂ INTAKE (MG) True	URINE N₂ (MG) Total	URINE N₂ (MG) Endogenous	URINE N₂ (MG) True	FÆCAL N₂ (MG) Total	FÆCAL N₂ (MG) Endogenous	FÆCAL N₂ (MG) Exogenous	Biological value	Mean
5 6	(I)	13 and 14	230 5	−7·5	13 6	243	123 8	115 4	98 1	41 8	56 3	40 1	31 7	8 4	51	
5 6	(II)	12 and 15	260 7	0 0	14 3	224	129 5	118 2	109 4	55 1	54 3	40 2	28 9	11 3	54	53
5 6	(III)	10 and 11	254 2	−4·7	14 3	230	129 9	118 0	108 7	55 9	52 8	39 9	28 0	11 9	55	
11 3	(IV)	13 and 14	248 7	1 8	14 6	216	263 8	242 1	209 9	42 8	168 1	55 7	34 0	21 7	31	
11 3	(V)	12 and 15	285 0	2 5	16 4	212	297 5	263 9	237 0	55 1	181 9	66 7	33 1	33 6	31	32
11 3	(VI)	10 and 11	274 7	7 0	16 9	225	305 6	274 5	238 1	55 9	182 2	64 2	33 1	31 1	33	
15 1	(VII)	13 and 14	228 2	4 3	16 5	240	398 8	373 9	317 1	41 8	275 3	63 3	38 4	24 9	26	
15 1	(VIII)	12 and 15	266 7	7 8	17 6	225	424 0	385 6	340 4	55 1	285 3	23 9	35 5	38 4	26	25
15 1	(IX)	10 and 11	250 0	8 0	17 0	232	410 4	373 0	342 9	55 9	287 0	70 7	33 3	37 4	23	

Table VI gives a comparative study of the biological values obtained by the three methods of calculation —

(1) Gives the values calculated on the assumption that metabolic nitrogen in the fæces is directly proportional to the food intake

(2) Gives the values calculated from the graphs .

(3) Gives the values in the calculation of which no allowance is made for the variation of metabolic nitrogen with varying food intake and the actual amount of nitrogen excreted in the fæces during the period of nitrogen-free feeding is taken as the endogenous fæcal output of a particular animal (Boas Fixsen and Jackson, *loc cit*).

TABLE VI

Comparison of the three methods of calculation

Protein (per cent)	Experiment number	BIOLOGICAL VALUE		
		(1)	(2)	(3)
		From the table	From the graph	Direct
(a) *Phaseolus mungo*				
5 8	(I)	63	61	58
5 8	(II)	65	62	59
5 8	(III)	62	62	59
11 6	(IV)	49	49	50
11 6	(V)	55	54	54
11 6	(VI)	52	53	53
15 0	(VII)	46	45	44
15 0	(VIII)	47	46	44
15 0	(IX)	43	43	42

TABLE VI—*concld*

Protein (per cent)	Experiment number	BIOLOGICAL VALUE		
		(1)	(2)	(3)
		From the table	From the graph	Direct

(*b*) *Lens esculenta*

Protein (per cent)	Experiment number	From the table	From the graph	Direct
5 6	(I)	51	49	48
5 6	(II)	54	53	51
5 6	(III)	55	54	53
11 3	(IV)	31	28	28
11 3	(V)	31	29	28
11 3	(VI)	33	32	31
15 1	(VII)	26	24	24
15 1	(VIII)	26	24	23
15 1	(IX)	23	22	21

(*c*) *Mixed diet (containing 10 per cent protein)*

		From the table	From the graph	Direct
	(I)	40	41	39
	(II)	34	34	35
	(III)	34	34	33

EFFECT OF CONCENTRATION OF PROTEIN ON THE BIOLOGICAL VALUE

A comparison of the results in Tables IV and V will show that as the concentration of protein in the ration increases, the biological value decreases   Thus, in the case of green gram the biological value of the protein at a 5 per cent level is 63 and the value falls to 52 when the protein concentration is 10 per cent and to 45 when the level of protein intake is increased to 15 per cent   Similarly in the case of lentil the biological value is 53 when the protein concentration is 5 per cent, at 10 per cent the value is 32 and at 15 per cent the value falls to 25

Thomas (*loc cit*) supposed that if sufficient fat and carbohydrate were supplied to cover the energy requirements of the organism the capacity of any protein to

replace the nitrogenous waste of the body tissues would remain constant at all levels of intake

Martin and Robison (*loc cit*) doubted the validity of this assumption, but in their own experiments with the proteins of whole wheat, same values were obtained at different concentrations of protein in the diet   Mitchel (1924b) carried out a large number of experiments on the biological value of proteins of milk, corn, oats, rice, yeast, potatoes, navy beans and the packing house by-product tankage with rations containing 5 per cent and 10 per cent of protein   With the exception of potatoes, the biological value was found smaller at the higher level

Boas Fixsen (*loc cit*) found the biological value of heated purified caseinogen to be independent of its concentration in the diet   But later on Boas Fixsen and Jackson (*loc cit*) stated that biological value of a protein diminished with increase in concentration of protein

It is noteworthy that this decrease in the biological value of proteins is related to protein concentration in the ration and not to the protein intake   This might be explained on the assumption that the greater the rate of influx of amino-acids to the tissues from the digestive tracts, the less the economy with which they are handled and utilized   If this were true, however, it would be expected that the utilization of dietary nitrogen in metabolism would be increased, at certain levels of intake at least, by increasing the number of feeds and thus causing a less rapid influx of amino-acids to the tissues   Chanutin and Mendel (1922) fed casein to dogs and found that the utilization of the protein was uneffected by the number of feeds   Mitchel (1924b), working with rats, obtained data similar to Chanutin and Mendel at low levels of intake   But with high protein ration, Mitchel's rats showed a higher utilization of nitrogen when the number of feeds was increased

The most probable explanation for this decrease in biological value with increasing concentration of protein appears to be that as the percentage of protein in the diet increases, the probability of its being oxidized in the tissues like carbohydrates and fats, also increases   In other words, with increasing concentration of protein the protein sparing action of the carbohydrate decreases

DIGESTIBILITY AND THE PROTEIN VALUE

The percentage digestibility of a protein may be defined as the number of parts of nitrogen digested out of 100 parts of the nitrogen of the food-stuffs ingested   It is evident that in calculating digestibility the metabolic nitrogen in the faeces must be taken into account to find out the exact amount of nitrogen actually digested   Much discussion has already been made on the question of metabolic nitrogen and in calculating the digestibility the same data as used in the computation of biological values have been used here

The term percentage digestibility may be expressed as —

$$100 \times \frac{\text{Food nitrogen digested}}{\text{Food nitrogen ingested}}$$

$$= 100 \times \frac{\text{Nitrogen intake} - (\text{Faecal nitrogen} - \text{Metabolic N}_2)}{\text{Nitrogen intake}}$$

The results are shown in Table VII —

TABLE VII

Digestibility

Protein (per cent)	Experiment number	FOOD NITROGEN		Percentage digestibility	Mean
		Intake	Digested		
(a) Phaseolus mungo					
5 8	(I)	139 6	130 6	93 5	
5 8	(II)	165 0	157 3	95 5	93
5 8	(III)	167 1	148 7	89 0	
11 6	(IV)	131 8	111 9	85 0	
11 6	(V)	159 9	137 4	86 0	86
11 6	(VI)	192 7	166 7	86 5	
15 0	(VII)	307 1	285 5	93 0	
15 0	(VIII)	387 2	362 0	93 5	93
15 0	(IX)	371 2	340 7	92 0	
(b) Lens esculenta					
5 6	(I)	123 8	115 4	93 2	
5 6	(II)	129 5	118 2	91 2	92
5 6	(III)	129 9	118 0	90 8	
11 3	(IV)	263 8	242 1	91 8	
11 3	(V)	297 5	263 9	88 7	90
11 3	(VI)	305 6	274 5	90 0	
15 1	(VII)	398 8	373 9	93 7	
15 1	(VIII)	424 0	385 6	91 0	92
15 1	(IX)	410 4	373 0	91 0	

The protein value of a food denotes the actual amount of protein utilized per 100 grammes of food ingested. It therefore depends as much upon the content of protein as upon the extent of its utilization in digestion and anabolism. By combining these three it can be estimated how much is ultimately available in covering the protein requirements of maintenance and growth. A method of obtaining numerical measures of the protein value of foods has been proposed by Mitchel *et al* (1927). According to him, the protein value of a food is given by the product of the protein content of the food, its digestibility and biological value. Or in other words—

$$\text{Protein value} = \text{Percentage of protein} \times \frac{\text{Biological value}}{100} \times \frac{\text{Percentage digestibility}}{100}$$

The protein values of the two pulses at different levels of intake are given in Table VIII —

TABLE VIII

Protein value

Protein (per cent)	Name of the pulse	Mean biological value	Mean digestibility	Protein content (per cent)	Protein value
5 8	Green gram	63	93	23 26	13 6
11 6	,, ,,	52	86	23 26	10 4
15 0	,, ,,	45	93	23 26	9 7
5 6	Lentil	53	92	22 66	11 1
11 3		32	90	22 66	6 5
15 1	,,	25	92	22 66	5 2

A comparison of the protein content, biological value, digestibility and protein value of the two pulses with the different articles of food would be interesting and is given in Table IX —

TABLE IX

Protein values of foods for maintenance and growth   Level of protein feeding 8 to 10 per cent

Food	Water content (per cent)	Protein content (per cent) (fresh basis)	Digestibility (corr) (per cent)	Biological value (per cent)	Protein value (per cent)
Whole egg	73 2	13 2	100	94	12 0
Milk	87 0	3 3	100	85	2 6
Egg white	86 2	12 3	100	83	10 0
Beef liver	71 2	20 4	90	77	14 9
Rolled oats	7 7	16 7	90	65	9 8
Whole wheat	11 4	13 8	91	67	7 1
White flour	12 8	10 8	100	52	4 3
Whole corn	10 3	7 5	95	60	3 0
Potato	78 3	2 2	78	67	0 8
Cocoa	4 6	21 6	38	37	1 6
Chocolate	5 9	12 9	38	37	0 4
Green gram	15 0	23 2	86	52	10 4
Lentil	15 1	22 6	90	32	6 5

SUPPLEMENTARY RELATION AMONG PROTEINS OF THE TWO PULSES

The biological value of a mixture of proteins is not necessarily the mean of the biological value of the individual proteins   Each protein fed at a given level of intake might be considered to consist of two fractions   One including the maximum amounts of several amino-acids that can be used to make good the loss of nitrogen catabolism and to cause the growth of the body tissues , the other fraction containing the remaining amino-acids but not in the right assortment for the purpose of synthesis and hence destined to be de-aminized   When the two proteins are fed simultaneously, the latter fractions of the two might supplement each other and make the assortment more complete for utilization   In such a case the biological value of the mixture would be greater than the mean of the biological value of each determined separately   It is evident that proteins having the same amino-acids deficiency will not exhibit any supplementary relation Obviously in demonstrating a true supplementary relation between two proteins or between the mixed proteins of two foods, it is required to measure the biological value of each protein separately and also in combination, the level of intake being the same in all cases

Sherman and his co-workers demonstrated a supplementary relation between the proteins of cereals and milk   Mitchel (1924c) showed the existence of supplementary relations between milk proteins and proteins of corn and between corn proteins and proteins of tankage

We carried out experiments with a diet containing 5 75 per cent of protein of both green gram and lentil making the total of 11 5 per cent   The results are shown in Table X —

TABLE X

Biological value of mixed diet (15 per cent)

Experiment number	Rat numbers	Average body weight (g)	Average change in body weight (g)	Food intake (g)	Non protein calorie intake (per kg body-weight)	N₂ INTAKE (MG)		URINE N₂ (MG)			FÆCAL NITROGEN (MG)			Biological value	Remarks
						Total	True	Total	Endogenous	True	Total	Endogenous	Exogenous		
(I)	21 and 22	228 5	—4	8 2	132	150 2	129 9	102 9	24 1	78 8	34 7	14 4	20 3	40	
(II)	23 and 25	288 5	—2 8	7 3	94	130 1	107 1	95 8	24 8	71 0	43 8	16 8	27 0	34	} Rejected for insufficient calorie intake
(III)	29 and 30	274 5	—4 5	8 7	114	160 2	134 0	110 6	21 7	88 9	44 0	17 8	26 1	34	

The mixed diet is ill-taken by the rats and it is very difficult to obtain sufficient intake of calories   Of the three sets of experiments mentioned in the table, the last two are to be rejected for insufficient calorie intake   It will be apparent that the biological value obtained with the mixed diet is 41 and the mean of the values obtained with green gram (52) and lentil (32) separately at 11 5 per cent level is 42   Obviously, there is no supplementary relation between the proteins of green gram and lentil and most probably the limiting amino-acids deficiency is the same in both of them

DISCUSSION

It will be seen from the above that though the two pulses contain the same percentage of protein and are almost equally digested the biological value of the green gram, as measured by the balance sheet method, is distinctly higher than that of the lentil at all levels of intake   It must be remembered that the biological value determined refers to the total nitrogen of the pulses and not to the total proteins, for a considerable fraction of the nitrogen content might be non-protein in form   This is specially true of the lentil   Our investigations on the proteins of the two pulses show that a considerable fraction of the nitrogen in the water extract of the lentil is non-protein in form   This might, to some extent, account for the lower biological value   It is not improbable, however, that this non-protein nitrogen might be present in the form of amino-acids and could be utilized for the maintenance and growth

Investigations on the proteins of the two pulses carried out by us further show that the proteins from lentil contain much less cystine than the green gram proteins   The relative deficiency of cystine may account for the lower biological value of lentil as compared with that of green gram

It should also be noted that the rate of decrease of biological value with increasing concentration of the proteins in the diet is much higher in the case of lentil than in the case of green gram   Thus from all standpoints the green gram is superior to lentil

Though the biological value of proteins of green gram (*cf* Table IX) is much less than that of whole egg, wheat and meat, the net protein value is not much less than these articles of food   This is undoubtedly due to its high protein content of good digestibility

SUMMARY.

1   The metabolic nitrogen of the fæces consists of two fractions   one dependent only on the body-weight and the other on the amount of food taken   It is suggested that the first fraction be called *nitrogen of the fasting metabolism*

2   The biological values of proteins of green gram at 5, 11 and 15 per cent levels of feeding are 63, 52 and 45 respectively   The corresponding values for the lentil are 53, 32 and 25

3   The biological value decreases with increase in the concentration of protein in the diet

4   The protein value of green gram is 10 4 and that of lentil 6 5 at a 10 per cent level of intake

5 There is no supplementary relation between the proteins of the two pulses

6 The green gram is superior to the lentil as a source of protein

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BIOLOGICAL VALUE OF THE PROTEINS OF GREEN GRAM (*PHASEOLUS MUNGO*) AND LENTIL (*LENS ESCULENTA*)

Part II

MEASURED BY THE GROWTH OF YOUNG RATS

BY

KALI PADA BASU, D Sc (Dac), PH D (Munich)

MADHAB CHANDRA NATH M Sc ,
Lady Tata Memorial Scholar

AND

MUHAMMAD OSMAN GHANI, M Sc

(*From the Biochemical Section, Chemical Laboratories, Dacca University*)

IN a previous communication (Basu, Nath and Ghani, 1936) were reported experiments in which the biological values of the proteins of green gram and lentil were determined by the balance sheet method of Thomas as improved by Mitchel With adult rats, the proteins of the green gram showed superiority over those of the lentil for the maintenance of nitrogen equilibrium It was next intended to investigate the value of the proteins of these two pulses in promoting the growth of young rats

Osborne and Mendel (1916) have proposed that the growth of young rats obtained with the substance under investigation as the only source of protein, gives the nutritive value of the proteins of the substance In this method, the gain in weight secured in rats of similar size in a period of feeding on a properly constituted ration is related to the protein actually consumed In comparing the biological values of different proteins by this method, however, Osborne and his co-workers

recommended that the rats of similar weight should be made to grow at equal rates, eating about the same amount of similarly constituted diets, the only varying factor in the diet being the nature and the proportion and hence the amount of protein The amount of protein ingested was taken to be inversely proportional to the biological value

Osborne, Mendel and Ferry (1919) later adopted the gain in weight per gramme of protein consumed as a measure of the growth-promoting value of the ration As the concentration of protein in the diet increased, the value of the ratio was at first found to increase reaching a maximum and then it declined They proposed to take the maximum value as the most correct measure of its biological value Later on, Osborne and Mendel (1920), in comparing the biological values of proteins of different cereals by this method, have simply taken the gain per gramme of protein ingested as a measure of the biological value when the different proteins were present at the same level

McCollum *et al* (1921), on the other hand insist that growth experiments can give a correct estimate of the biological values of proteins, only when such experiments include observations on longevity and reproduction Osborne and Mendel (1920) fully considered this view-point and concluded that growth experiments for a limited period, conducted under proper control can give reliable information about the nutritive adequacy of proteins

Mitchel (1924) has thoroughly discussed this question and is of opinion that nitrogen balance studies supplemented by growth experiments, as recommended by Osborne and Mendel are sufficient to determine the adequacy or otherwise of proteins in nutrition

Hoagland and Snider (1926, 1927) also have applied this numerical method in extensive studies of the proteins of animal foods Morgan (1931) has determined the effect of heat upon the biological value of cereal proteins and casein by this method

Recently Boas Fixsen *et al* (1934) have applied this method to determine the comparative biological values of whole wheat whole maize and maize gluten

In this investigation the gain in weight per gramme of protein ingested at the same level of intake of protein and at comparable intake of calories, has been taken as the criterion in comparing the biological values of the proteins of the two pulses Experiments have been carried out with rations containing different percentages of proteins

The composition of the diets is shown in Table II in Part I of this paper

EXPERIMENTAL PROCEDURE

Young albino rats (bred in this laboratory) of about 40 g weight were used Each rat was kept in a separate cage of the same type as used in vitamin B_1 estimation To prevent loss of food, the cage was allowed to rest on a sheet of blotting paper This also served the purpose of absorbing the urine and facilitated the collection of scattered food Water was supplied from a glass bulb kept inverted in the cage The test substances were fed at 5 per cent, 10 per cent, and 15 per cent levels in each case

PLATE XXVIII
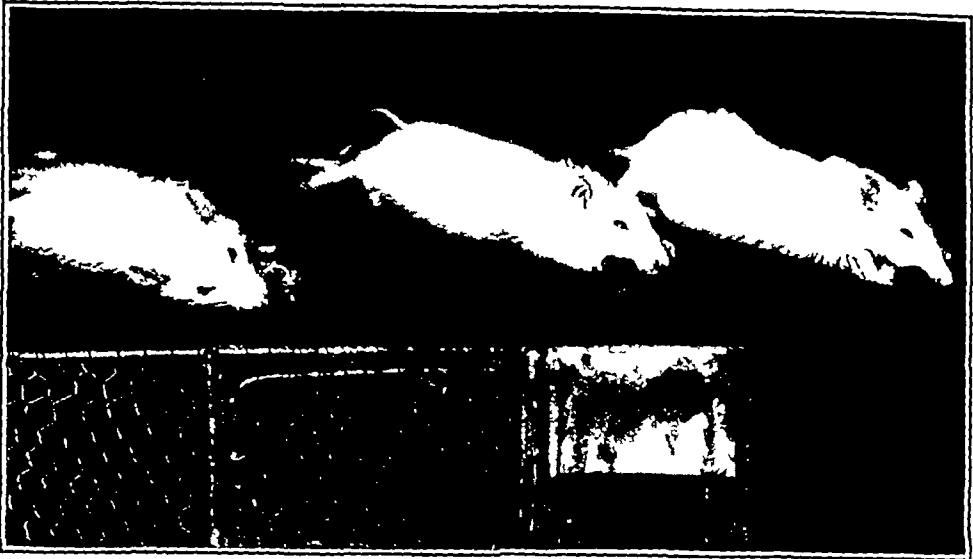
PHOTOGRAPH 1 —From right to left   Rat on normal diet, rat on green gram diet (protein
15 per cent) and rat on lentil diet (protein 15 per cent)
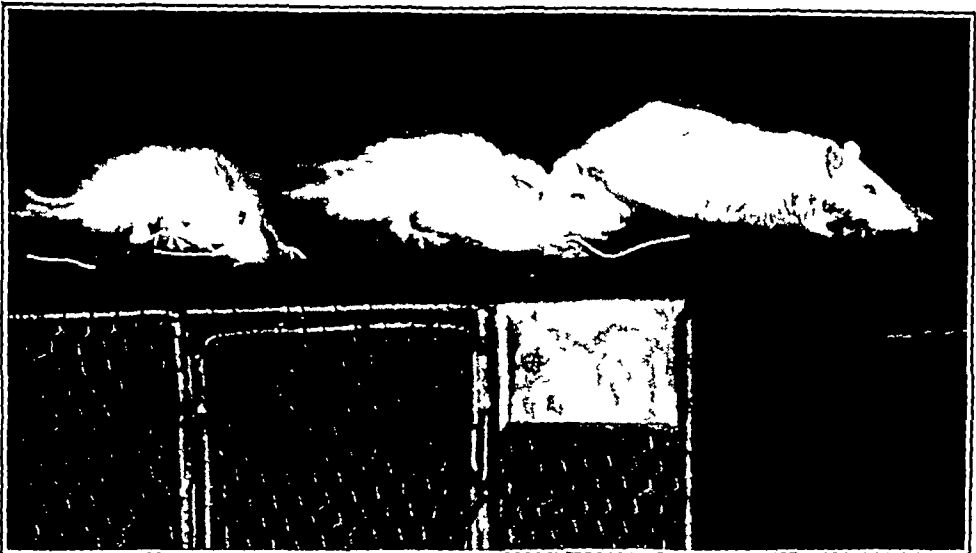
PHOTOGRAPH 2 —From right to left   Rat on normal diet, rat on green gram diet (protein
10 per cent) and rat on lentil diet (protein 10 per cent)

PHOTOGRAPH 3 —From right to left   Rat on normal diet, rat on green gram diet (protein
5 per cent) and rat on lentil diet (protein 5 per cent)

Five rats were kept on each diet   The different diets were distributed as evenly as possible between litter mates   The daily ration was weighed out for each rat, added to the residue of the previous day, cooked in water and given in the form of a thick paste   The daily amount given to each rat was adjusted to the day's requirement of the animal and thus a large residue was avoided

At the end of every week, the residue in the feeding cup and scattered food were collected dried in an oven at 100°C and weighed   Thus the food intake as well as the protein intake for the week were calculated out   The weight of the rats were taken every week   The experiments lasted for 9 weeks   The first week was treated as a preliminary period and no record of food intake was taken   The calculation was made on the result of the subsequent 8 weeks

To provide the vitamin B complex, each rat was supplied with a weekly supplement of 2 c c of a dilute solution of a yeast preparation   Each rat thus received 6 4 mg of nitrogen from this source per week   The total nitrogen intake from this source amounted to 51 2 mg in the whole experiment and was therefore, negligible

The nutritive values were then calculated from the total protein intake and the total gain in weight in 8 weeks   For comparison, the values, taking into consideration the first 4 weeks only, were also calculated

The change in body-weight was plotted against time in weeks for each rat Four rats representing 4 litters used in these experiments were also kept on a normal diet consisting of whole wheat and milk supplemented occasionally by cod-liver oil and marmite and the rate of growth was observed as in other cases   These were taken as the standard animals kept on a normal diet and were used for comparison

Results

The performances of the individual rats are represented graphically in Figs 1, 2, 3 5, 6 and 7

The complete results are summarized in Tables I and II

General Condition of the Rats on Different Diets

The animals receiving 5 per cent diets of both the varieties became very much emaciated and looked very unhealthy, after about a week only   Besides failure of growth, marked loss of fur was also observed   This was very much pronounced in the case of the lentil diet   In the case of the lentil diet containing 10 per cent, as well as that containing 15 per cent protein, the rats showed loss of fur though not so prominently as those on the 5 per cent diet   In the case of green gram 15 per cent diet there was no loss of fur though the general condition of the fur as regards its silkiness and smoothness seemed to be affected   The 10 per cent diet of green gram caused loss of fur, though only very slightly The general condition of rats on different diets is shown in the three photographs on Plate XXVIII

J, MR

It will be seen from the above results that when the growth of young rats is taken as the criterion, the proteins of the green gram are immensly superior to those of the lentil Thus at 10 per cent of protein in the diet, the ratio $\frac{gain\ in\ weight}{protein\ consumed}$ after eight weeks of feeding is 1 16 for the green gram and 0 59 for the lentil While 15 per cent concentration of the protein, the corresponding values are 1 23 and 0 94 The superiority of the proteins of green gram over those of the lentil is also evident from the nature of the feeding curves With only 5 per cent of protein in the diet, the rats kept on green gram just maintain their weight without showing any growth, while those on lentil showed a decline in weight, as will be seen from the curves in Figs 6 and 7 Moreover, the general condition of the rats on the green gram diet was much better than that of the rats kept on the lentil diet at the same concentration of protein in the ration The fact that the rats on lentil generally showed loss of fur together with our unpublished investigations on the proteins of lentil which revealed a deficiency of the latter in cystine, point to the fact that lack of cystine is the cause of this inferior performance To test this, 0 2 per cent cystine was added to the diets containing 10 per cent and 15 per cent protein from the lentil and these were given to rats 89, 102, 87 and 93 after the 9th week The result was growth of fur and also an increase in the rate of gain in weight Supplemented with 0 2 per cent cystine, the 15 per cent diet led to almost a complete recovery of the fur in a period of two weeks, whereas in the case of the 10 per cent diet a distinct resumption of the growth of hair was noted The effects of addition of cystine are shown in Figs 2 and 5

EFFECT OF THE PERCENTAGE OF PROTEIN IN THE DIET ON THE

RATIO $\frac{GAIN\ IN\ WEIGHT}{PROTEIN\ CONSUMED}$

It will be seen from Tables I and II that for the same cereal, as the concentration of protein in the diet increases, the value of the ratio also increases Thus for the green gram, as the concentration increases from 10 per cent to 15 per cent, the value of the ratio also increases from 1 16 to 1 23, while the corresponding increase in the case of lentil is from 0 59 to 0 94

This is in accordance with the previous observation of Osborne and his co-workers Mitchel (1924, 1928) has offered an explanation for the fact that for any given protein the value of this ratio at first increases with increasing concentration of protein in the diet According to him no account is taken in this expression of the protein required for maintenance apart from that required for growth The former is evidently a greater proportion of the total intake when this is small In Fig 8 increase in weight in eight weeks is plotted against protein intake for each rat receiving 10 per cent and 15 per cent of protein from the green gram and lentil and also on a mixed diet containing 10 per cent of protein, 5 per cent from each pulse It will be found that the experimental points all lie grouped about a straight line which when produced backwards cuts the abscissa at a point corresponding to about 9 g of protein This amount of protein should represent the protein requirement for maintenance for 8 weeks of rats varying from 50 g to 80 g in weight

FIGURE 1.

FIGURE 2

FIGURE 3.

Body-weight (g.).

Weeks.

FIGURE 4

114
113
112
116

— Mixed diet (Green gram &
Lentil, protein 10 %)

Body-weight (g.).

117 (male)

118 (female)

——— Lentil diet (Protein 10 %)
— — Normal diet.

82
87
+.2 % cystine
101
+.2% cystine
108
93
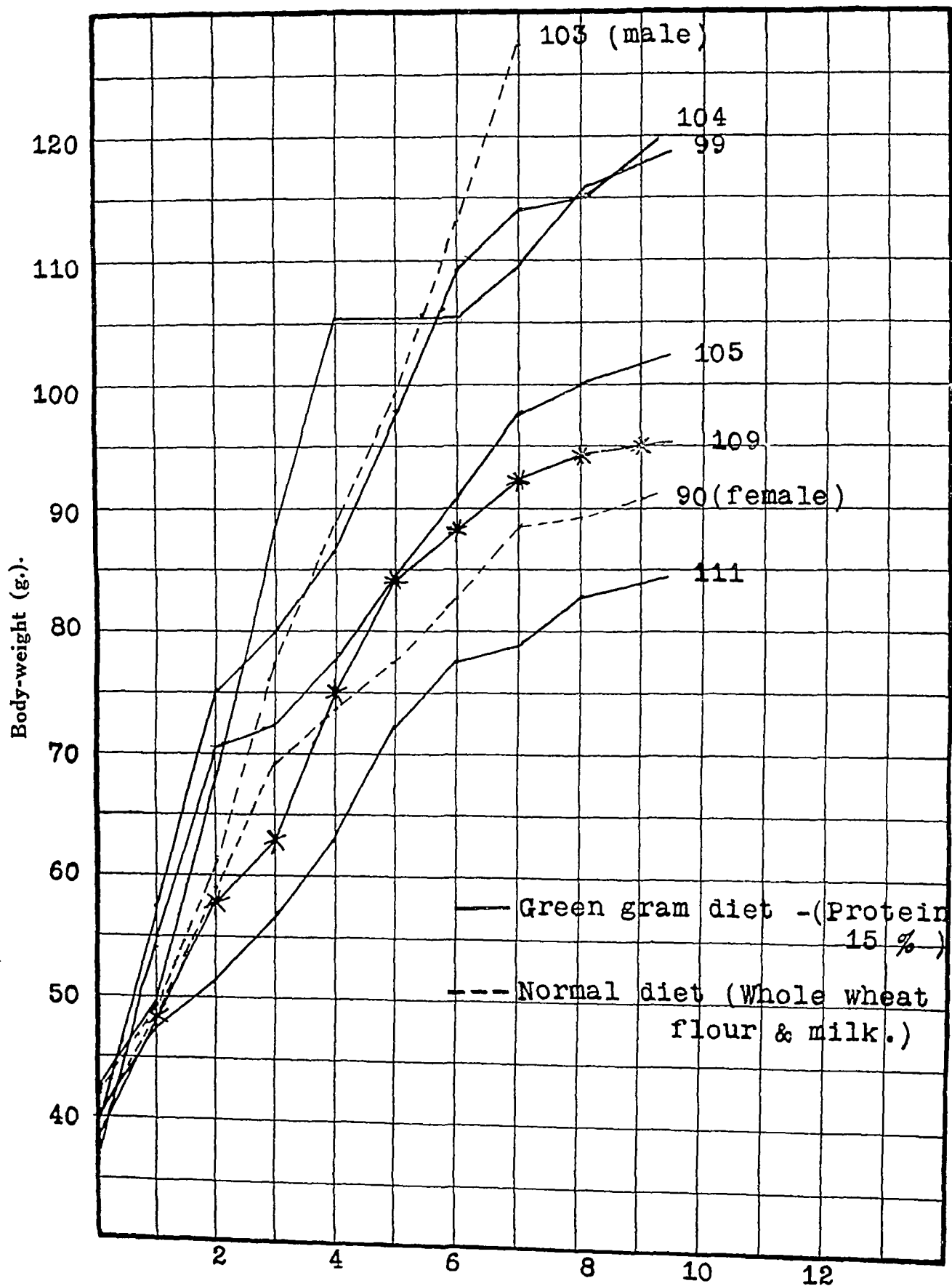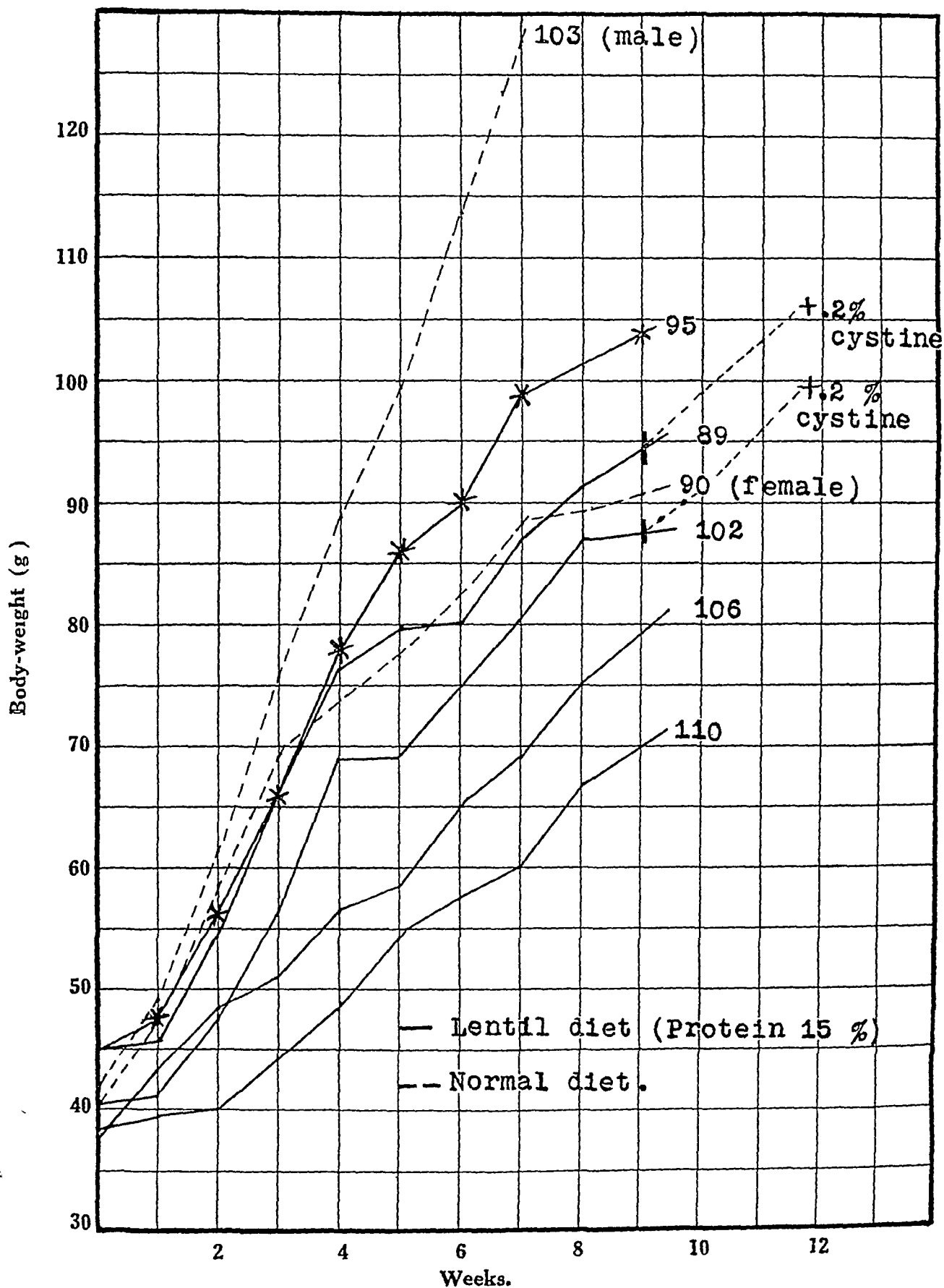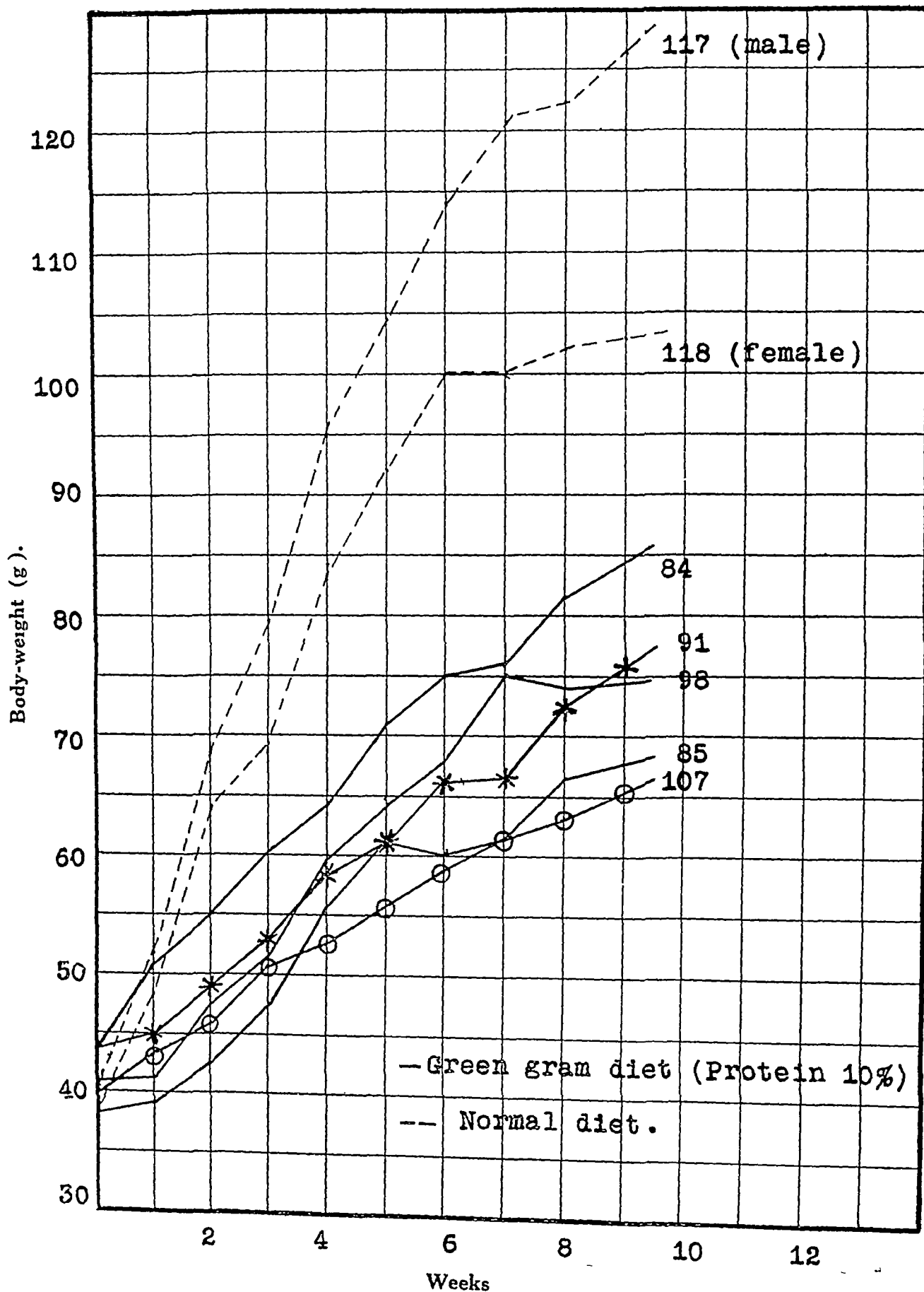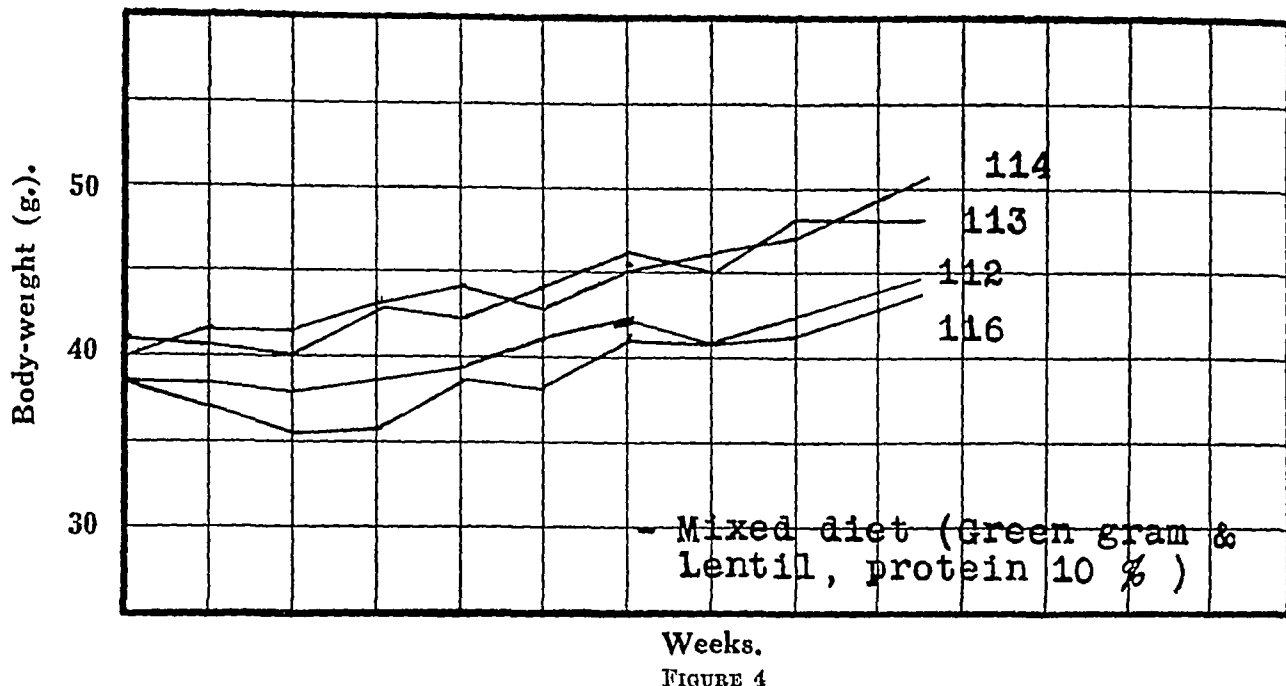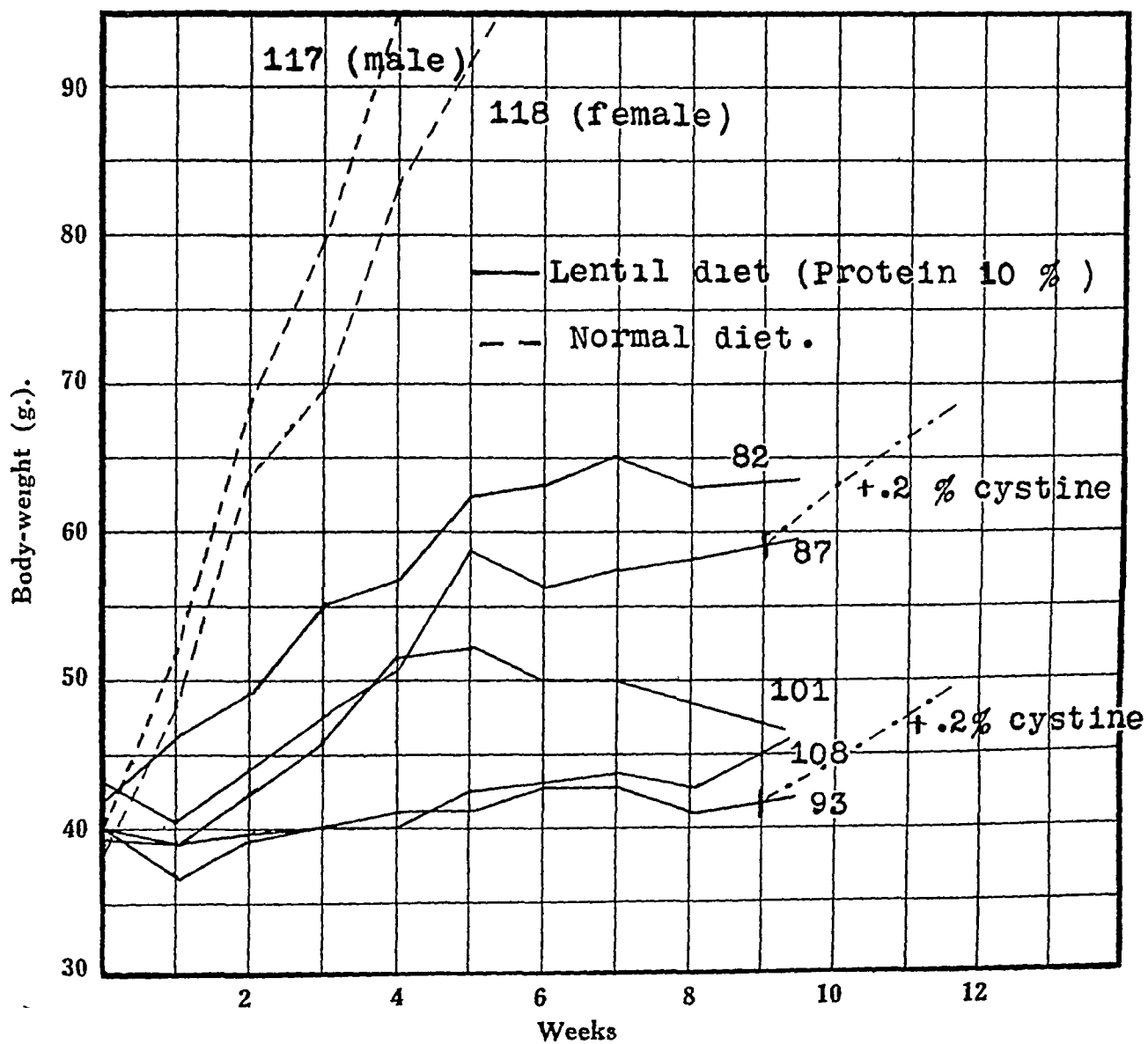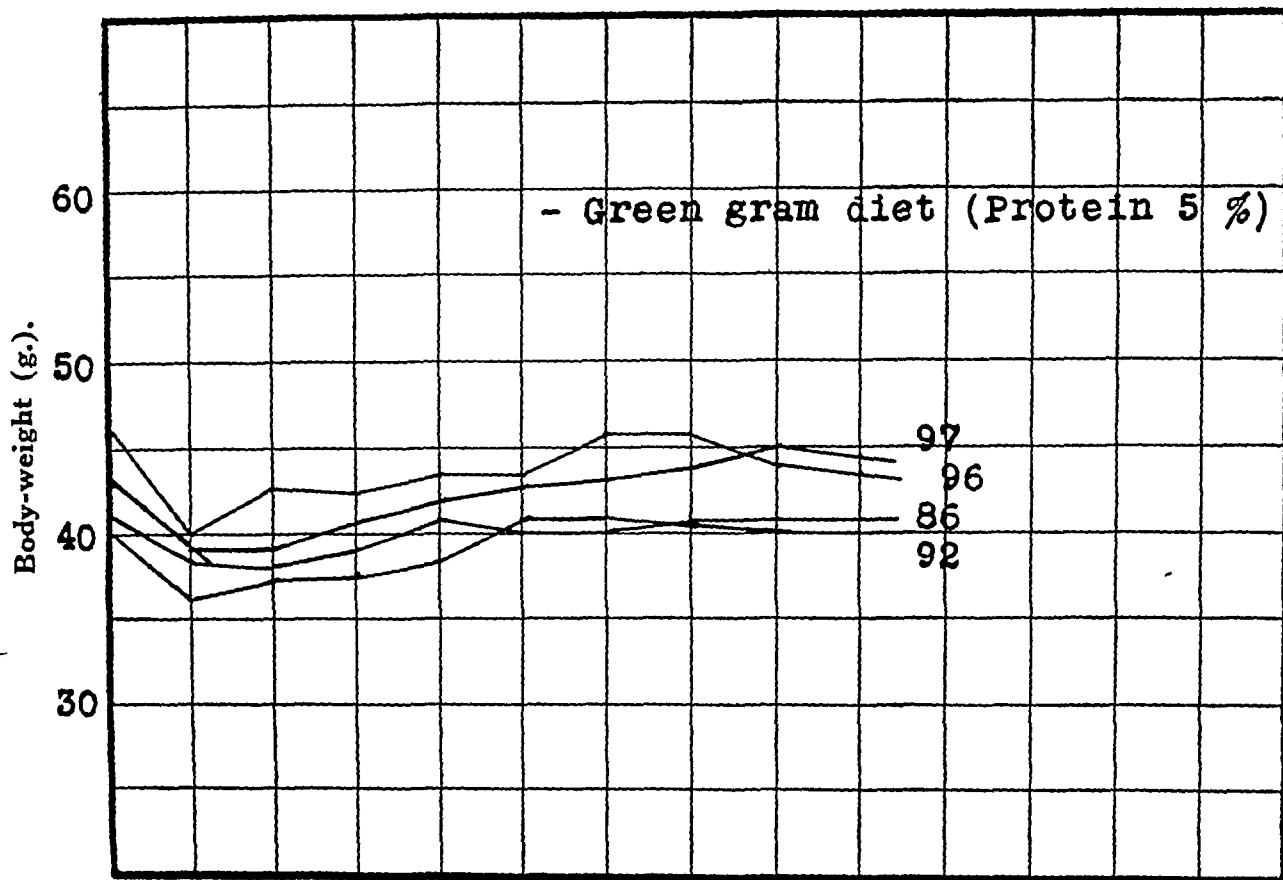
Weeks

FIGURE 5
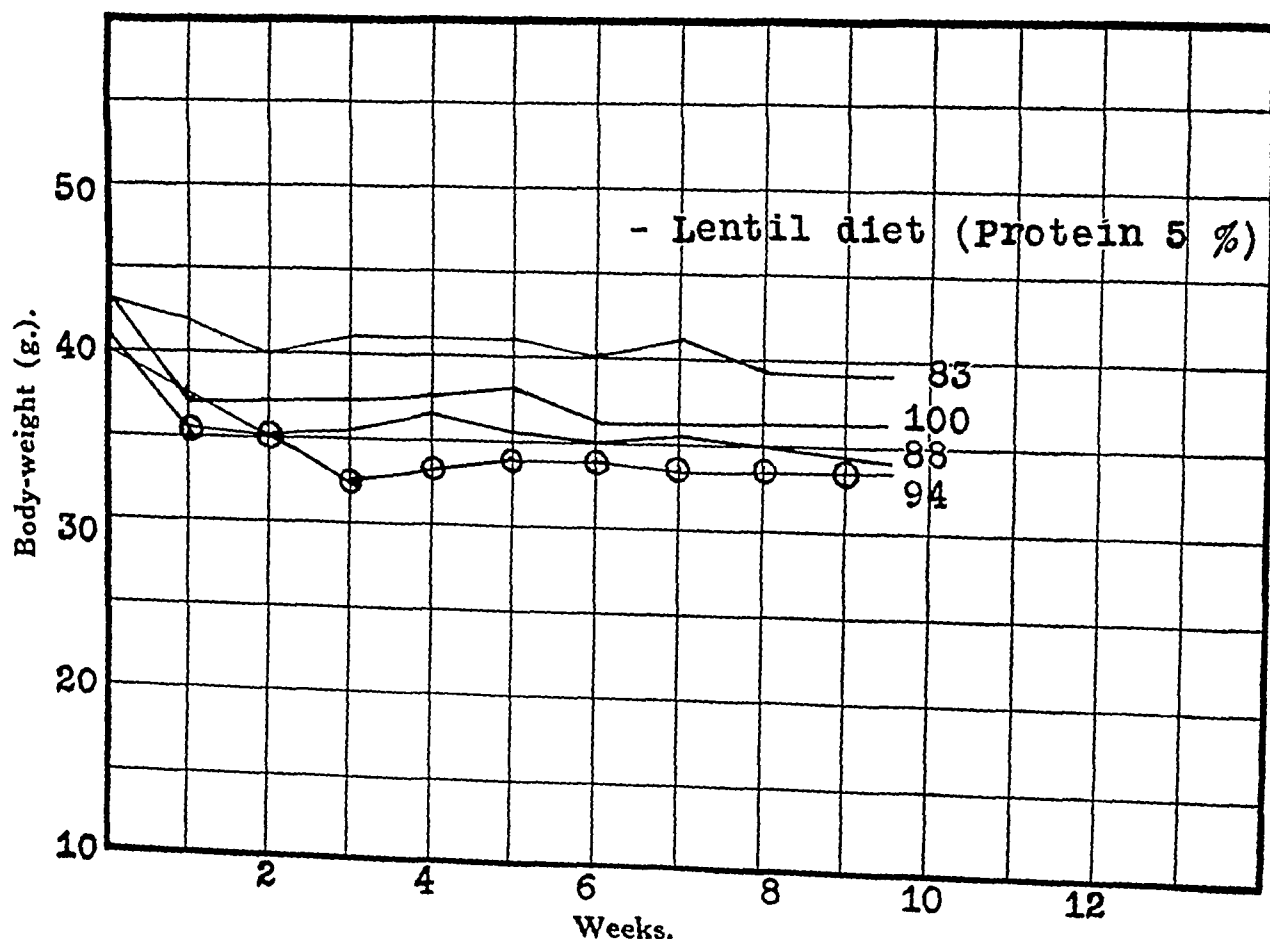
Weeks.

FIGURE 6

Weeks.

FIGURE 7
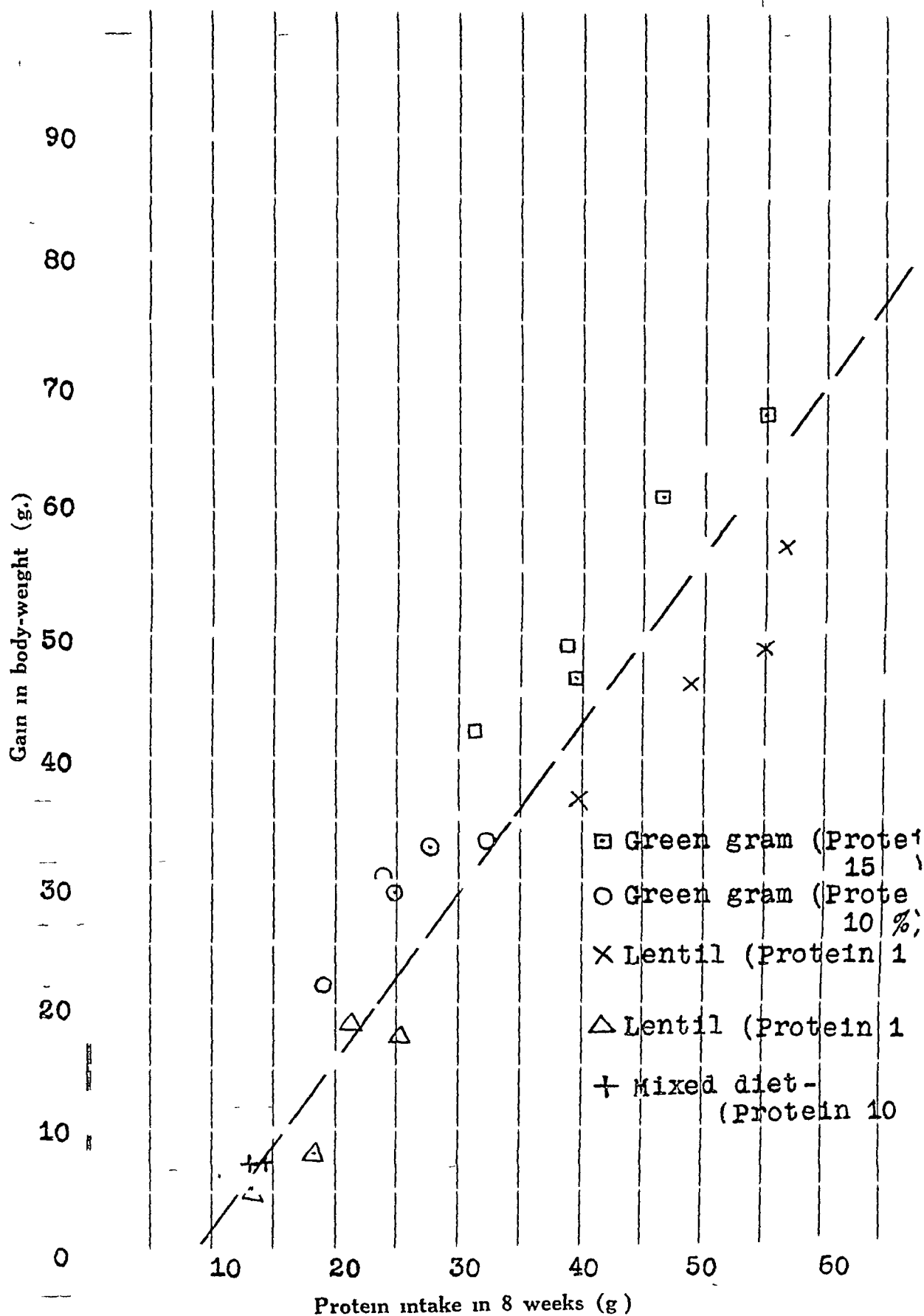
FIGURE 8

TABLE I

Biological values of Phaseolus mungo

Protein content (per cent)	Rat number	Litter number	Sex	4 WEEKS						8 WEEKS						
				Initial weight (g)	Food intake (g)	Protein intake (g)	Gain in weight (g)	Biological value = gain in weight / protein intake	Average biological value	Food intake (g)	Protein intake (g)	Calorie intake (total)	Gain (g)	Biological value = gain in weight / protein intake	Average biological value
15 2	88	D	Male	50 0	206 0	31 30	55 5	1 77	1 62	358 5	54 49	1,409	67 5	1 24	1 23
15 2	104	E	Female	57 5	160 4	24 30	39 5	1 63		306 7	46 62	1,205	61 0	1 31	
15 2	105	E	,,	55 0	137 7	20 94	29 5	1 41		259 5	39 45	1,020	46 5	1 18	
15 2	109	F	,,	48 0	137 0	20 84	36 5	1 75		255 1	38 76	1,002	49 0	1 26	
15 2	111	G	Male	47 0	119 6	16 14	25 0	1 55		240 7	32 45	946	37 0	1 14	
10 2	84	A	Female	51 0	159 5	16 23	20 0	1 23	1 48	170	32 26	1,309	33 5	1 04	1 16
10 2	85	B	,,	39 0	123 3	12 52	22 0	1 75		244 6	24 89	1,003	29 0	1 16	
10 2	91	C	Male	45 0	112 5	11 48	16 0	1 39		234 2	23 90	967	30 5	1 28	
10 2	98	D	,,	41 5	121 8	12 42	22 5	1 81		260 7	27 60	1,077	33 0	1 20	
10 2	107	E	Female	43 5	98 0	10 01	12 0	1 20		194 5	19 86	803	22 0	1 11	

TABLE II

Biological values of Lens esculenta

Protein content (per cent)	Rat number	Litter number	Sex	Initial weight (g)	4 WEEKS					8 WEEKS					
					Food intake (g)	Protein intake (g)	Gain in weight (g)	Biological value = gain in weight / protein intake	Average biological value	Food intake (g)	Protein intake (g)	Calorie intake (total)	Gain (g)	Biological value = gain in weight / protein intake	Average biological value
15 1	89	B	Female	45 5	188 6	28 48	34 0	1 19	1 12	364 1	54 98	1,460	48 5	0 88	0 94
15 1	95	C	,,	47 0	193 1	29 16	38 5	1 32		375 2	56 66	1,504	57 0	1 00	
15 1	102	D	Male	11 0	153 7	23 18	28 0	1 20		324 2	48 90	1,300	46 0	0 94	
15 1	106	E	Female	43 5	118 0	17 82	15 5	0 88		262 4	30 60	1,052	36 5	0 92	
15 1	110	F	Male	39 5	100 2	15 12	15 0	0 99		213 7	32 19	856	30 5	0 95	
9 8	82	A	Male	46 0	137 0	13 49	16 5	1 22	1 05	256 0	25 19	1,057	17 5	0 69	0 59
9 8	87	B	,,	40 5	119 2	11 88	18 0	1 50		218 2	21 62	901	18 5	0 85	
9 8	93	C	Female	37 0	74 3	7 27	4 5	0 62		134 2	13 13	554	5 0	0 38	
9 8	101	D	Male	39 0	109 0	10 64	13 0	1 22		188 1	18 40	777	8 0	0 44	
9 8	108	E	Female	38 5	58 6	5 74	4 0	0 69		115 2	11 28	476	6 5	0 58	

The ratio $\dfrac{\text{gain in weight}}{\text{protein consumed—protein required for maintenance}} = X_2$ should be constant irrespective of the concentration of the protein in the diet   But in our experiments the amount of protein taken is not very great compared with the protein required for maintenance and hence this ratio has been calculated only for those cases where the food intake was sufficiently great   Table III gives the corrected growth value of green gram and lentil at a 15 per cent concentration   It should be noted that Boas Fixsen *et al* (*loc cit*) have recently obtained 10 g of protein as the maintenance requirement for similar rats for a period of 9 weeks with the whole wheat and maize as the sources of protein —

TABLE III

Corrected nutritive values

		$X_1 = \dfrac{\text{Gain in weight}}{\text{Protein intake}}$	$X_2 = \dfrac{\text{Gain in weight}}{\text{Protein intake} - 9}$
15 per cent green gram	(1)	1 24	1 48
	(2)	1 31	1 62
	(3)	1 18	1 52
	(4)	1 26	1 64
	(5)	1 14	1 58
15 per cent lentil	(1)	0 88	1 05
	(2)	1 00	1 20
	(3)	0 94	1 15
	(4)	0 92	1 19
	(5)	0 95	1 31

THE EFFECT OF SEX UPON GROWTH PER GRAMME OF PROTEIN

Hoagland and Snider (1926) have raised the question of effect of sex upon the rate of growth per gramme of protein taken and have tried to show from their data that the ratio obtained by this method is larger for male rats than for the female

ones   The experiments of Morgan (*loc cit*), on the other hand, do not wholly bear out this contention and according to the data of Morgan this ratio is independent of sex

Mitchel (1924) is of opinion that more of the protein eaten is used for growth by the male rats than by the female ones

The effect of sex on the observed nutritive value is shown in Table IV   It will be seen that with a single exception (in the case of green gram diet containing 15 per cent protein) the male rats give a slightly higher value than the female ones —

TABLE IV

Comparison of gain per gramme of protein eaten by male and female rats

Type of diet	Protein level (per cent)	Number of rats used	Average gain per gramme of protein
Green gram	15 2	2 males	1 19
	15 2	3 females	1 25
	10 2	2 males	1 24
	10 2	3 females	1 10
Lentil	15 1	2 males	0 95
	15 1	3 females	0 93
	9 8	3 males	0 66
	9 8	2 females	0 48

THE EFFECT OF DURATION OF EXPERIMENTS ON GAIN PER GRAMME OF PROTEIN

It will be seen from Tables I and II that as the period of experiment increases from 4 to 8 weeks the growth per gramme of protein diminishes Similar results have been obtained by the previous investigators in this field (*cf* Osborne and Mendel, 1920)

SUPPLEMENTARY RELATION BETWEEN THE PROTEINS OF THE TWO PULSES

Growth experiments on a mixed diet containing 10 per cent protein, including 5 per cent from each pulse, were carried out to find out if there was any

supplementary relation between the proteins of the two pulses   The results are represented graphically in Fig 4 and are summarized in Table V —

TABLE V

Biological values of mixed diet (10 *per cent protein*)

Rat number	Litter number	Sex	Total intake		Initial weight	Gain in weight	Gain per g protein	Average biological value
			Protein	Calorie				
112	F	Female	12 36	511	38 5	6 0	0 48	
113	F	Male	13 52	560	40 5	7 5	0 55	
114	G	Female	14 52	601	41 5	7 5	0 52	0 54
115	F	,,	14 44	598	37 5	10 0	0 69	
116	G	,,	12 93	535	37 0	6 0	0 46	

The biological value obtained with the mixed diet is 0 54 whereas those obtained with green gram and lentil separately at the same level of intake (10 per cent) are 1 16 and 0 59 respectively   It is apparent that there is absolutely no supplementary relation between the proteins of these two pulses   This observation agrees with the results obtained in the nitrogen balance sheet method reported already by us (Basu, Nath and Ghani, *loc cit*)

CONCLUSION

From the results of this investigation together with the results already reported in Part I of this paper, it is evident that both as regards replacing waste tissues and formation of new ones *Phaseolus mungo* is immensely superior to *Lens esculenta* The cause of this difference must be sought in the amino-acid make up of the proteins from the two sources and obviously isolation and chemical analysis of the proteins of these pulses would furnish valuable information on this point This will form the subject-matter of a future communication

SUMMARY

1   The biological values of the proteins of green gram and lentil have been determined by the numerical method of Osborne, Mendel and Ferry   Growth per gramme of protein ingested is 1 23 at 15 per cent concentration of protein in the diet and 1 16 at 10 per cent concentration, in the case of green gram   The corresponding values in the case of lentil are 0 94 at the 15 per cent and 0 59 at the 10 per cent level of intake

2   With 5 per cent of protein from these sources, the rats on the green gram just maintain their weights, while those on the lentil lose weight

3   For the growth of young rats, green gram proteins are immensely superior to those from lentil   Rats on green gram ration containing 15 per cent protein grow almost as efficiently as normal rats kept on a diet of milk and whole wheat

4   Rats on lentil showed loss of fur   This could be remedied by the addition of 0 2 per cent cystine to the diet

5   There seems to be a tendency of the male rats to give higher biological values than those given by the female ones, under similar conditions

6   There is no supplementary relation between the proteins of the two pulses

7   The maintenance requirement for rats varying from 50 grammes to 80 grammes of body-weight appears to be 9 g of protein from these two pulses for a period of 8 weeks

REFERENCES

Basu, Nath and Ghani (1936)	*Ind Jour Med Res* , **23,** p 789
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Morgan (1931)	*Jour Biol Chem* , **90,** p 771
Osborne and Mendel (1916)	*Ibid* , **26,** p 1
Idem (1920)	*Ibid* , **41,** p 275
Osborne, Mendel and Ferry (1919)	*Ibid* , **37,** p 223

ENZYMIC DIGESTIBILITY OF PULSES ACTION OF SALIVARY AND PANCREATIC AMYLASE AND OF THE PROTEOLYTIC ENZYMES PEPSIN AND TRYPSIN

BY

KALI PADA BASU, D SC (Dac), PH D (Munich),

AND

SATIPRASAD MUKHERJEE, M SC

(From the Biochemical Section, Chemical Laboratories, Dacca University)

PULSES form a constant ingredient of the daily diet of the Indian For the vegetarians they form the chief source of proteins The readiness with which their proteins and also their carbohydrates are digested in the body in the presence of the different substances of which they are composed should be different The value of these food-stuffs should to some extent depend on the rate of their digestibility Some *in vitro* experiments have been carried out and the results of these experiments should throw some light on the *in vivo* processes

The digestibility of the most common pulses, the green gram (*Phaseolus mungo*), lentil (*Lens esculenta*), peas (*Pisum arvense*), gram (*Cicer arietinum*) and also of two varieties of soya bean, the black and the white, forms the subject matter of the present investigation The two varieties of the soya bean were very kindly supplied by the Economic Botanist to the Government of Bengal

EXPERIMENTAL

The digestibility of the proteins has been determined with pepsin (Merck) and trypsin (Messrs Carnrick & Co , U S A) activated by cystine The starch hydrolysis has been carried out with saliva and also with pancreatic amylases.

The technique employed was the same as in the previous investigations on the digestibility of the different varieties of Bengal rice by Basu and Mukherjee (1935) The results are given in the following tables —

<div align="center">TABLE I</div>

Showing the variation in the digestibility of different pulses by the proteolytic enzymes, pepsin and trypsin at temperature 40°C and pH 2 and pH 8 5 respectively

Variety	Time in hours	Amount of alcoholic KOH (N/25) required in c c	
		By pepsin	By trypsin
Lentil (*Lens esculenta*)	1	0 565	0 819
	3	0 904	1 469
Peas (*Pisum arvense*)	1	0 537	0 791
	3	0 819	1 130
Green gram (*Phaseolus mungo*)	1	0 338	0 451
	3	0 564	0 789
Gram (*Cicer arietinum*)	1	0 376	0 647
	3	0 537	1 075
Soya bean (white)	1	0 577	0 577
	3	0 787	0 840
Soya bean (black) .	1	0 565	0 631
	3	0 739	0 810
Rice non polished	Mean for 3 hours	0 748	0 827
Rice polished		0 787	0 900

TABLE II

Showing the variation in the digestibility of different pulses by the salivary as well as the pancreatic amylases

Variety	Time in minutes	MG of REDUCING SUGAR	
		Salivary digestion	Pancreatic digestion
Lentil (*Lens esculenta*)	4	8 37	7 67
	8	10 76	9 67
	15	12 56	11 85
Peas (*Pisum arvense*)	4	6 62	8 37
	8	10 01	10 76
	15	10 61	11 85
Green gram (*Phaseolus mungo*)	4	6 62	7 32
	8	9 05	9 32
	15	11 05	10 81
Gram (*Cicer arietinum*)	4	6 02	6 82
	8	9 12	8 50
	15	9 96	8 96
Soya bean (white)	4	5 58	6 27
	8	8 67	9 76
	15	10 06	11 16
Soya bean (black)	4	5 48	6 88
	8	8 58	9 06
	15	9 97	11 15

DISCUSSION

The pulse proteins, it will be seen, are more readily hydrolysed by trypsin than by pepsin Towards pepsin, gram is the least and lentil the most readily digestible, whereas green gram and lentil are the least and most digestible respectively in case of tryptic digestion

Considering the two most common pulses, the green gram and the lentil, investigations in this laboratory (Basu, Nath and Ghani, 1936) show that the biological value of the proteins of green gram at 11 per cent level of intake is 52,

the corresponding figure for the lentil being 32 The percentage digestibility for the proteins of both the pulses is almost the same in two cases, the value being 90 The superiority of the green gram over the lentil as a source of protein is to some extent diminished by the fact that the proteins of the lentil are much more easily digested than those of the green gram

Although the pulses contain about $3\frac{1}{2}$ times as much proteins as rice it will be seen that the amount of amino-acids obtained after three hours' hydrolysis is of the same order in the case of rice and the pulses Thus rice protein appears to be more readily digestible than the pulse protein Moreover, the biological value of rice protein is also quite high being 86 (Mitchel 1924) The rice protein appears to be of a better quality than the pulse protein

The rate of digestibility of soya bean proteins is of the same order as that of the pulses studied Our investigations show that soya bean contains a very high percentage of proteins 46 10 per cent Rose and McLeod (1925), experimenting on four young women found that on a soya bean diet the faecal nitrogen was high showing a low biological value of the proteins, but that nitrogen equilibrium was maintained on a very low intake Thus a more extensive use of the soya bean as a food-stuff especially by the adult people in this country, can be recommended The nutritive value of the proteins of the soya bean is under investigation in this laboratory

So far as the digestion of the carbohydrates is concerned the lentil starch appears to be the most digestible towards salivary amylase Towards the pancreatic amylase all the cereal-starches investigated are almost equally digestible with the exception of those of the green gram and gram which are the least digestible ones

Our thanks are due to Professor J C Ghosh for his interest in this investigation

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BASU and MUKHERJEE (1936)	*Ind Jour Med Res*	**23,**	p 777
BASU, NATH and GHANI (1936)	*Ibid ,*	**23,**	p 789
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ROSE and McLEOD (1925)	*Ibid ,*	**66,**	p 847

THE RELATION BETWEEN THE COMPOSITION OF THE DIET AND THE URINARY EXCRETION OF ASCORBIC ACID

BY

RAMA KANTA CHAKRABORTY[+]

AND

AMRITA NATH ROY

(*From the Department of Biochemistry and Nutrition, Indian Institute for Medical Research, Calcutta*)

THE investigation was undertaken with a view to find the relation, if any, between the ingestion of high-carbohydrate, high-protein and high-fat diets and the urinary excretion of ascorbic acid It was considered that this might throw some light on the function of vitamin C in relation to metabolism and on the need of vitamin C by different communities habitually living on different diets Meat (mutton) and casein were used as two different sources of protein and butter was used as the source of fat

The Tillman's method of estimating ascorbic acid by titration against 2 6 dichlorophenol-indophenol as developed by Harris and Ray (1933) was employed (*see also* Chakraborty, 1935) In all cases twenty-four hours' urine was examined The urine was titrated immediately after excretion whenever possible but the urine voided at night and in the following morning were preserved by adding sufficient glacial acetic acid to the urine so that the strength of the solution was approximately 10 per cent The urine that was preserved at night was titrated within 8 to 9 hours of excretion and the urine that was voided early in the morning was titrated within 2 or 3 hours of excretion 0 05 c c of 0 01 M solution of the dye was used to which 1 c c glacial acetic acid was added (Ghosh and Guha, 1935) and the titrations against urine were completed within one minute The evaluation of ascorbic acid in urine by this method is based on the assumption that the reducing substance in urine consists solely of ascorbic acid—an assumption which is supported by the evidence of the work of Harris and Ray (1935)

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* Lady Tata Memorial Scholar

The subjects of the experiments were two healthy Bengali young men, R K C (case I) and A N R (case II)   They started by taking the usual high-carbohydrate Bengali diet, consisting, on an average, of 672 g boiled rice, 112 g dhal (boiled aqueous decoction of pulses), 90 g fish curry and 160 g vegetable curry approximately in case I and 742 g boiled rice, 123 g dhal, 100 g fish curry and 146 g vegetable curry approximately in case II

The ascorbic acid content of the urine collected over 24 hours was estimated for four days   The experimental subjects were then placed on a high-protein diet for seven days, in which meat replaced an approximately equal quantity of rice, the proportion of meat being gradually raised from 115 g on the first two days to 461 g on the 7th day   The daily urinary excretion of ascorbic acid was estimated on this diet   The subjects were then put on the usual high-carbohydrate Bengali diet for seven days, by which time the urinary output of ascorbic acid decreased The subjects were then placed on a high-fat diet for six days in which butter fat replaced approximately an equal quantity of rice   The proportion of fat was raised from 58 g on the first day to 230 g on the 5th and 6th days and the daily excretion of ascorbic acid was noted   The subjects were then placed again on the ordinary Bengali high-carbohydrate diet for 7 days to allow the urinary output of ascorbic acid to fall   The subjects were then fed on a high-protein diet for five days, in which casein replaced an approximately equal quantity of rice, the proportion of casein being increased gradually from 115 g on the first day to 403 g on the 5th day and the daily output of ascorbic acid in urine was estimated

The figures are given in Tables I, II, III and IV   It has to be mentioned that, during the whole course of the experiment, cooked food was taken and fresh fruits were scrupulously avoided so that the results might not be complicated by the consumption of unknown quantities of ascorbic acid   As the experiments were carried out during the hot months, the volumes of urine voided were usually low

TABLE I

Experiment with the usual high-carbohydrate Bengali diet

Date	Normal quantity of diet taken per day (approximate figures in g)	Volume of urine passed in 24 hours (c c)	Total quantity of ascorbic acid (mg) excreted in urine during 24 hours
		Case I	
20-4-35	921	571	8 09
21-4-35	1,036	833	9 61
22-4-35	921	864	10 82
23-4-35	1,151	811	9 65
		Average	9 54

TABLE I—*concld*

Date	Normal quantity of diet taken per day (approximate figures in g)	Volume of urine passed in 24 hours (c c)	Total quantity of ascorbic acid (mg) excreted in urine during 24 hours
		Case II	
20–4–35	1,213	475	9 42
21–4–35	978	483	9 76
22–4–35	1,151	490	10 12
23–4–35	1,208	454	9 69
		Average	9 75

TABLE II

Experiment with high-protein (meat) diet

Date	Total quantity of diet taken per day (approximate figures in g)	Quantity of high protein diet taken per day (g)	Total volume of urine (c c) excreted per day	Total quantity of ascorbic acid (mg) per day
		Case I		
24–4–35	921	115	743	13 25
25–4–35	979	115	824	12 96
26–4–35	921	230	1,082	11 91
27–4–35	863	230	824	12 08
28–4–35	979	230	642	10 26
29–4–35	921	345	708	11 15
30–4–35	1,036	461	851	12 91
			Average	12 07
		Case II		
24–4–35	1,036	115	565	10 46
25–4–35	691	115	606	10 56
26–4–35	806	230	633	14 09
27–4–35	806	230	634	12 43
28–4–35	749	230	427	8 19
29–4–35	749	345	337	8 94
30–4–35	863	461	632	12 87
			Average	11 08

TABLE III

Experiments with high-fat (butter fat) diet

Date	Total quantity of diet taken per day (approximate figures in g)	Total quantity of of high fat diet taken per day (g)	Total volume of urine (c c) excreted per day	Total quantity of ascorbic acid (mg) excreted per day
		Case I		
8–5–35	806	58	523	12 61
9–5–35	921	115	505	13 30
10–5–35	749	115	572	14 08
11–5–35	749	173	587	14 62
12–5–35	518	230	487	14 83
13–5–35	518	230	585	15 05
			Average	14 09
		Case II		
8–5–35	979	58	316	9 92
9–5–35*	979	115	357	11 75
10–5–35	863	173	352	17 70
11–5–35	806	230	344	17 44
12–5–35	691	230	387	17 18
			Average	14 80

* These experiments with high fat diet were commenced when the urinary excretion of ascorbic acid per day had dropped to 10 21 mg and 8 51 mg in cases I and II respectively after an interval of 7 days on the ordinary high carbohydrate diet

TABLE IV

Experiment* with high-protein (casein) diet

Date	Total quantity of diet taken per day (approximate figures in g)	Total quantity of high casein diet taken per day (g)	Total volume of urine (c c) excreted per day	Total quantity of ascorbic acid (mg) excreted per day
		Case I		
21–5–35	806	115	641	13 66
22–5–35	806	173	635	12 88
23–5–35	863	173	923	12 90
24–5–35	806	288	703	14 26
25–5–35	749	403	908	19 54
			Average	14 65
		Case II		
21–5–35	863	115	528	12 91
22–5–35	863	173	417	12 84
23–5–35	749	173	571	12 30
24–5–35	863	288	539	12 47
25–5–35	806	403	633	15 10
			Average	13 12

* These experiments were commenced when the urinary excretion of ascorbic acid per day had fallen to 9 57 mg and 10 46 mg in cases I and II respectively after an interval of 7 days on the usual high carbohydrate diet

DISCUSSION

It will be observed that in case I the average daily urinary output of ascorbic acid, 9 54 mg, on the usual Bengali high-carbohydrate diet rose to 12 07 mg on the meat diet, an increase of 26 5 per cent. It fell to 10 21 mg on the subject being placed again on the usual high-carbohydrate diet, but rose again to 14 69 mg on the high-fat diet, an increase of 38 0 per cent. On the resumption of the ordinary diet it fell to 9 57 mg, which rose again to 14 65 mg on the subject being placed on a casein diet an increase of 53 1 per cent

In case II the usual high-carbohydrate diet gave an ascorbic acid value of 9 75 mg, which rose to 11 08 mg on a meat diet, an increase of 13 64 per cent A period of the usual high-carbohydrate diet brought the level down to 8 51 mg,

which rose again to 14 80 mg on a high-fat diet, an increase of 74 0 per cent On being diverted to the usual high-carbohydrate diet, the level fell to 10 46 mg, which rose again to 13 12 mg on a casein diet, an increase of 25 4 per cent

It appears from these results that there is a considerable increase in the urinary excretion of ascorbic acid in both cases I and II on a high-fat diet as well as on two high-protein diets one containing casein and the other meat It is not improbable that the higher figures for the urinary excretion of ascorbic acid in England, as given by Harris and Ray (1935), are due to the greater consumption of meat in that country The relatively lower figures obtained by us do not necessarily indicate vitamin C sub-nutrition, as in some unpublished experiments we have found that a sharp peak is produced in the urinary excretion of ascorbic acid on the ingestion of extra vitamin C (*cf* Harris and Ray, 1935)

The nature of the chemical events, which underlie the increased excretion of ascorbic acid in urine on high-fat and high-protein diets is under investigation It appears possible that ascorbic acid has some special rôle in the metabolism of fat and protein

The possibility of the presence of other reducing substance or substances in urine, which might complicate these results, is under investigation

SUMMARY

In dietetic experiments with two healthy Bengali males it has been found that a high-fat diet (with butter as the source of fat) as well as two high-protein diets (one containing casein and the other meat as the source of protein) produce a significant increase in the total daily urinary excretion of ascorbic acid, as determined titrimetrically

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THE ADSORPTION OF ANTIGENS BY ANTI-BODIES OR VICE VERSA

Part II

BY

B N GHOSH, D sc,

University College of Science, Calcutta,

92, Upper Circular Road

In a previous paper (Ghosh, 1935) it has been shown that the mutual interaction of antigens with anti-bodies can be quantitatively accounted for by equations based on the assumption that the antigens are preferentially adsorbed by their respective anti-bodies or vice versa   In that paper although the possibility of existence of complicated types of adsorption was recognized, the cases considered in detail were comparatively simple   These were (I) where an antigen or anti-body particle occupies one active point on the surface of the adsorbent and (II) where such a particle occupies two active points on the surface of the adsorbent   In the present paper some examples will be considered in which the anti-body samples appear to consist of two types of particles, one type consisting of particles each of which occupies one active point and the other type consisting of particles each of which occupies two active points on the surface of the antigens   Such examples therefore constitute a mixed type of adsorption involving simultaneously the reactions contemplated under cases I and II in the previous paper

The adsorption isotherm for such a mixed type of adsorption may be deduced in the following way   Let us assume that a sample of anti-body consists of two types of particles as described above   Let the particles, each of which occupies one active point on the surface of the antigen be denoted by *particles of type I* and the particles each of which occupies two active points be denoted by *particles of type II*

In T units of this sample of anti-body let T_1 units be contributed by particles of type I and T_2 units by particles of type II

Then $T = T_1 + T_2$

and $\frac{T_1}{T_2} = \gamma$ a constant for this particular sample

Again

$$\gamma + 1 = \frac{T_1}{T_2} + 1 = \frac{T_1 + T_2}{T_2} = \frac{T}{T_2}$$

$$T_2 = \frac{T}{\gamma + 1}$$

Also $T_1 = T - T_2 = T - \frac{T}{\gamma + 1} = \frac{\gamma T}{\gamma + 1}$

Let us now suppose that T units of the anti-body sample are added to C units of the antigen and that the volume of the system is always brought to a constant value   When equilibrium is attained let P units of anti-body be adsorbed   To this total adsorption of P units of anti-body, let the contribution of the particles of type I be P_1 and that of particles of type II be P_2 units, i e, $P = P_1 + P_2$

Again let one unit of antigen contain x particles each of average surface area S sq cm and let n be the number of active points per unit area of the surface where adsorption is possible   Then the total number of such active points for C units of antigen is $CxSn$

If P_1 units of particles of type I occupy K_1P_1 active points and P_2 units of particles of type II occupy K_2P_2 active points at equilibrium, then the number of free active points is equal to $(CxSn - K_1P_1 - K_2P_2)$

So the fraction of the surface which is free is equal to

$$\frac{CN - K_1P_1 - K_2P_2}{CN} \quad (\text{putting } xSn = N)$$

(I) Therefore the rate of adsorption of particles of type I

$$= \frac{K_3 \ (CN - K_1P_1 - K_2P_2) \ (T_1 - P_1)}{CN}$$

and the rate of their desorption

$$= \frac{K_4 P_1}{CN}$$

At equilibrium

$$\frac{K_3 \ (CN - K_1P_1 - K_2P_2) \ (T_1 - P_1)}{CN} = \frac{K_4 P_1}{CN} \qquad \text{.. } (a)$$

Now if K_1P_1 and K_2P_2 are very small compared to CN they can be neglected without introducing any appreciable error in the calculation

Furthermore if C is maintained constant then equation (a) reduces to the form

$$K_5 \ (T_1 - P_1) = K_4 P_1$$

$$\text{or, } (T_1 - P_1) = Ka P_1 \ \left(\text{putting } Ka = \frac{K_4}{K_5}\right)$$

$$\text{or, } P_1 = \frac{T_1}{Ka + 1}$$

But $T_1 = \frac{\gamma}{\gamma + 1} T$

$$\therefore P_1 = \left(\frac{1}{Ka + 1}\right) \left(\frac{\gamma}{\gamma + 1}\right) T \qquad . \ (1)$$

(*II*) The rate of adsorption of particles of type II, since each of them occupies two neighbouring points on the surface, is equal to

$$\frac{K' (CN - K_1P_1 - K_2P_2)^2 (T_2 - P_2)}{(CN)^2}$$

and the rate of their desorption is equal to

$$\frac{K'' (P_2)^n}{(CN)^n}$$

Therefore at equilibrium

$$\frac{K' (CN - K_1P_1 - K_2P_2)^n (T_2 - P_2)}{(CN)^n} = \frac{K'' (P_2)^2}{(CN)^2}$$

Now neglecting K_1P_1 and K_2P_2 for reasons already stated we have

$$K' (CN)^2 (T_2 - P_2) = K'' (P_2)^2$$

or, $\frac{K'}{K''} (CN)^2 (T_2 - P_2) = (P_2)^2$.   (*b*)

when C is maintained constant $(CN)^2$ is constant

Therefore putting K_x for $\frac{K'}{K''} (CN)^2$ we can write equation (*b*) in the form

$$K_x (T_2 - P_2) = P_2^2$$

or, $P_2^2 + K_x P_2 - K_x T_2 = 0$

Solving for P_2 and taking the real root, we have

$$P_2 = \frac{- K_x + \sqrt{K_x^2 + 4K_x T_2}}{2}$$

But $T_2 = \frac{T}{\gamma + 1}$

Substituting this value of T_2 in the above equation, we have

$$P_2 = \frac{- K_x + \sqrt{K_x^2 + 4 \frac{K_x T}{\gamma + 1}}}{2} \tag{2}$$

Therefore the total number of units P of anti-body adsorbed is given by the equation

$$P = P_1 + P_2 = \left(\frac{1}{K_a + 1}\right)\frac{\gamma}{\gamma + 1} T + \frac{-K_x + \sqrt{K_x^2 + 4 K_x T (\gamma + 1)}}{2} \tag{3}$$

Since γ, K_x and K_a are constants the above expression can be written as

$$P = K_0 T + \frac{- K_x + \sqrt{K_x^2 + 4 K_y T}}{2} \tag{4}$$

Where $K_0 = \frac{1}{K_a + 1} \left(\frac{\gamma}{\gamma + 1}\right)$ and $K_y = \frac{K_x}{\gamma + 1}$

It should be noted that $K_x > K_y$ and that the right hand side terms of equation (4) contains T as the only variable, K_0, K_x and K_y being constants

Therefore for different known values of T it is possible to calculate from equation (4) the corresponding values of P, and therefore also of $(T - P)$ provided the constants K_0, K_x and K_y are evaluated first

ADSORPTION OF AGGLUTININ BY TYPHOID BACILLI

Eisenberg and Volk (1902) have measured the adsorption by typhoid bacilli of their specific agglutinin   The agglutinin serum was diluted with physiological saline so as to obtain solutions of different requisite strengths   A given volume of each of these solutions was mixed separately with a fixed volume of a suspension of typhoid bacilli contained in a series of tubes   After allowing sufficient time for the attainment of equilibrium, the tubes were centrifugalized and the amount of free agglutinin in each was estimated   The difference between the number of units of agglutinin taken and the number of units left free gives the number of units of agglutinin adsorbed   In Table I the observed values of the units of agglutinin adsorbed are compared with the values calculated from equation (4)   The constants $K_o = 0\,32$, $K_x = 2,000$ and $K_y = 1,250$ were evaluated from three sets of experimental data corresponding to three different values of T (the number of units of agglutinin taken)   It may be pointed out that under the experimental conditions stated above, amount of free agglutinin less than one unit cannot be determined

Considering the limits of error possible in such experiments, the agreement between the observed and calculated values appears to be quite good

TABLE I.

$$K_o = 0\,32, \quad K_x = 2,000, \quad K_y = 1,250$$

Units of agglutinin added (T)	UNITS OF AGGLUTININ ADSORBED	
	Observed	Calculated from equation (4)
2	2	1 9
20	20	19 0
40	40	37 8
200	180	182
400	340	352
2,000	1,500	1,510
10,000	6,500	5,870
20,000	11,000	10,500

In Table II are compared the values of free agglutinin as observed by Eisenberg and Volk with those calculated from equation (4) and also from the empirical adsorption isotherm $B = Ka^n$ used by Arrhenius (1907)   In this equation B represents the number of units of agglutinin adsorbed, a the number of units of agglutinin free and K and n are constants, $K = 24\ 7$, $n = \frac{2}{3}$ It may be mentioned that in the experimental procedure adopted by Eisenberg and Volk the amount of free agglutinin just equal to or less than one unit could not be estimated and had to be recorded as equal to zero   Taking this fact into consideration the values calculated from equation (4) are in as good agreement with the observed values as those calculated from Arrhenius' equation

<div align="center">

TABLE II

Typhus agglutinin

$K_o = 0\ 32,\quad K_x = 2,000,\quad K_y = 1,250$

</div>

Units of agglutinin added (T)	UNITS OF AGGLUTININ FREE		
	Observed	Calculated from equation (4)	Calculated from Arrhenius' equation
2	0	0 1	0 02
20	0	1 0	0 70
40	0	2 2	2 10
200	20	18	19 70
400	60	48	51
2,000	500	490	478
10,000	3,500	4,130	4,269
20,000	9,000	9,500	9,400

ADSORPTION OF AGGLUTININ BY CHOLERA VIBRIOS

The adsorption by cholera vibrios of their specific agglutinin was also measured by Eisenberg and Volk (1902)   The experimental procedure was the same as in the

adsorption of agglutinin by typhoid bacilli   In Table III the observed values of the number of units of agglutinin adsorbed corresponding to different values of the units (T) of agglutinin taken are compared with those calculated from equation (4) The constants K_o, K_x and K_y were found to be 0 31, 1,250 and 800 respectively

TABLE III

Cholera agglutinin

$$K_o = 0\ 31, \quad K_x = 1,250, \quad K_y = 800$$

Units of agglutinin added (T)	Units of agglutinin adsorbed	
	Observed	Calculated from equation (4)
2	2	1 92
20	20	19 10
40	38	37 50
67	60	62 00
200	120	179
2,000	1,300	1,400
11,000	6,500	5,860
20,000	10,000	10,380

In Table IV are recorded the values of free cholera agglutinin observed by Eisenberg and Volk together with those calculated from equation (4) and also from the empirical equation $B = K_a{}^n$ used by Arrhenius where B, a, K and n have the same significance as stated before   In this case $K = 19$ and $n = \frac{2}{3}$ It should be noted that in these experiments also the amount of free agglutinin just equal to or less than one unit could not be estimated and had to be put equal to zero   The agreement between the observed and calculated values is fairly good except where the observed value of free agglutinin is 80 and the calculated value is 21 only

Eisenberg and Volk, however, admit in their memoir the possibility of this particular observation being vitiated by accidental error

<div align="center">TABLE IV</div>

$$K_o = 0\ 31, \quad K_x = 1,250, \quad K_y = 800$$

Units of agglutinin added (T')	Units of agglutinin free		
	Observed	Calculated from equation (4)	Calculated from Arrhenius' equation
2	0	0 08	0 03
20	0	0 90	1 00
40	2	2 50	2 80
67	7	5 00	5 60
200	80?	21	2?
2,000	700	600	620
11,000	4,500	5,140	5,260
20,000	10,000	10,380	10,750

ADSORPTION BY ERYTHROCYTES OF OX OF THEIR SPECIFIC HÆMOLYSIN.

Morgenroth and Arrhenius (1907) carried out some experiments on the adsorption of hæmolysin produced by injecting erythrocytes of the ox into the veins of a rabbit The immune serum of the rabbit was diluted with physiological saline so as to obtain solutions of different strengths These were cooled to a low temperature and a given volume of each of these solutions was mixed separately with a fixed volume of a suspension of ox erythrocytes contained in a series of tubes The tubes were kept at a low temperature and after about an hour they were centrifugalized The number of units of hæmolysin free in the supernatant fluid in each tube was then determined In Table V are recorded the values of the units of hæmolysin adsorbed as observed by the above authors together with the values calculated from equation (4) The constants K_o, K_x and K_y are found to be 0 245,

2,330, and 1,450 respectively   It will be noticed that the agreement between the observed and calculated values is fairly good

<div align="center">

TABLE V

$$K_o = 0\ 245, \quad K_x = 2,330, \quad K_y = 1,450$$

</div>

Units of hæmolysin added ($\varGamma$)	Units of hæmolysin adsorbed	
	Observed	Calculated from equation (4)
250	226	208
330	275	271
670	500	523
1,330	850	960
2,700	1,710	1,790
5,000	3,070	2,990
10,000	5,800	5,270
16,700	7,820	7,980
33,000	13,900	13,440

ADSORPTION BY ERYTHROCYTES OF SHEEP OF THEIR SPECIFIC HÆMOLYSIN

Morgenroth and Arrhenius (*loc cit*) measured the adsorption of hæmolysin (heated at 56°C for some time) by sheep's erythrocytes   The hæmolysin was prepared by injecting erythrocytes of sheep into a goat   The experimental procedure was the same as described previously   In Table VI the observed number of units of hæmolysin adsorbed are compared with the values calculated from equation (4)   The constants, K_o, K_x and K_y, were found to be 0 42, 5,150 and 2,750 respectively   It will be noticed that there is fairly good agreement between the observed and calculated values.

TABLE VI

$$K_o = 0\,42, \quad K_x = 5{,}150, \quad K_y = 2{,}750$$

Units of hæmolysin added (T)	UNITS OF HÆMOLYSIN ADSORBED	
	Observed	Calculated from equation (4)
200	189 5	189
400	374	373
800	723	723
1,600	1,384	1,410
3,200	2,400	2,690
6,400	5,230	5,040
12,800	9,420	9,270

In Table VII are recorded Morgenroth's data on the number of units of hæmolysin left free along with the values calculated from equation (4) and from the empirical equation $B = K_a{}^n$ used by Arrhenius   In this equation B, a, K and n have the same significance as mentioned already, K was equal to 39 5 and n was equal to $\frac{2}{3}$

TABLE VII

$$K_o = 0\,42, \quad K_x = 5{,}150, \quad K_y = 2{,}750$$

Units of hæmolysin added (T)	UNITS OF HÆMOLYSIN FREE		
	Observed	Calculated from equation (4)	Calculated from Arrhenius' equation
200	10 5	11	10 5
400	26	27	28 8
800	77	77	78 4
1,600	216	190	207
3,200	800	510	550
6,400	1,170	1,370	1,420
12,800	3,380	3,530	3,570

CONCLUSION

The data recorded in Tables I to VII show that the values calculated from equation (4) deduced by the author are in agreement with the observed values within the limits of experimental error

SUMMARY

In continuation of the author's previous work it has been shown in this paper that when the antigen or the anti-body samples consist of two types of particles, an equation can be deduced on the basis of the assumptions stated below, which quantitatively accounts for the observed facts

(1) The anti-body sample consists of two types of particles such that a particle of one type occupies one active point and that a particle of the other type occupies two active points on the surface of the adsorbent

(2) That the amount of adsorbent taken is very large so that even at the highest number of units of antigen or anti-body used only a small fraction of the surface of the adsorbent is covered

The equation is of the form

$$P = K_0 T + \frac{-K_1 + \sqrt{K_2^2 + 4K_3\, T}}{2}$$

The above equation has been tested in a number of cases and has been found fairly satisfactory

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THE PROTEUS GROUP OBSERVATIONS ON 25 STRAINS MAINTAINED AT THE KING INSTITUTE MADRAS [*]

BY

S R PANDIT

(*From the King Institute, Guindy, Madras*)

THE genus *Proteus* includes organisms isolated from various sources   Hauser (1885) isolated them from putrefying meat and was the first to give a full description of this group of organisms   Stools, urine, pus, waters of rivers and pools, sewage and soil are some of the other sources from which these organisms have been isolated The isolation of certain strains—X strains—by Weil and Felix (1916, 1917) from typhus cases and the observation that these organisms were agglutinated in very high dilutions of typhus sera provide additional interest to this group   A classification of the group was first attempted by Hauser (*loc cit*)   He subdivided it into 3 species on the basis of morphology, rate of liquefaction of gelatine and production of indol   Others have attempted classification on serological lines   Much of the earlier work on serological classification loses its importance owing to the fact that the difference between flagellar ('H') and somatic ('O') antigens was not recognized It will be recalled that it was with organisms of the Proteus group that Weil and Felix (1917) showed, by separating the 'O' and 'H' variants, the difference between the two antigens   Wenner and Rettger (1919) studied 73 strains and proposed a classification on the basis of maltose fermentation and suggested the name *Proteus vulgaris* to those that fermented and *Pr mirabilis* to those that did not ferment this carbohydrate   Moltke (1927) studied 194 strains, including X strains   In his monograph he gives an exhaustive bibliography and refers to previous work on the subject   He found that the strains could be divided into two sharp groups on the basis of maltose fermentation   One hundred and thirty-six strains did not ferment maltose   The maltose fermenting strains were divided into several sub-groups according to the fermentation of salicin, rhamnose, melezitose and arbutin   All the X strains belonged to one group   There was no correlation between fermentation reactions and serological grouping   By the qualitative antigenic analysis of 'H' and 'O' antigens, Moltke showed that by direct agglutination swarming forms divided themselves into 3 large main groups besides a number of minor groups

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and by absorption of agglutinins the 3 main groups could be divided into sub-groups

Taylor (1928) studied 53 strains of Proteus isolated from urine, pus, stools and lung   Of these, only 3 fermented maltose   Nine agglutinating sera were prepared and absorption tests were done, but no distinction was made between ' H ' and ' O ' agglutination

Yacob (1932) studied 35 Proteus strains including X2, X19 and Kingsbury strains   He prepared 4 agglutinating sera   No absorption tests were, however, carried out

Coming to Proteus X strains, Weil and Felix (1918) consider, after examining 126 strains of ordinary Proteus, that a complete difference exists between the ' O ' receptors of these strains and those of their so-called X2 strains on the one hand and X19 on the other   These latter are, therefore, serologically separate species and are distinct from the ordinary Proteus organisms

Felix and Rhodes (1931) comparing Kingsbury and X strains come to the conclusion that the former is a variant of Proteus X19 and that while its ' O ' antigen is completely different from that of X2 and X19, there is a relationship as regards the ' H ' antigen, between Kingsbury and X strains and also with the ordinary Proteus strains

In the present study 25 strains of Proteus have been examined   Two of them—National and X19—are X19 strains obtained from the National Collection of Type Cultures at different times and Kingsbury and Kuala Lampur are strains obtained from the Federated Malay States   R A M C , Shanmugam, Jayalakshmi and 4809 are strains of which the source of origin is not known, though it appears as if R A M C is an X strain obtained from the Royal Army Medical College   The remaining 17 strains were isolated from stools and urine sent for examination to this laboratory   Brinkhurst and Mrs K   were isolated from urine and the remaining 15 from fæces   The property of ' swarming ' of Proteus was utilized to obtain pure cultures in most cases   The water of condensation at the bottom of a fresh agar slope was inoculated with the material   If a fresh agar tube was not available, a tube of agar medium was melted down and then re-sloped   After a few hours of incubation or the next morning, a thin hazy growth was usually seen to spread up the surface of the medium in waves or ripples, if Proteus was present   Sub-culture from the edge of the growth invariably gave a pure culture of Proteus

MORPHOLOGY, CULTURAL CHARACTERS, BIOCHEMICAL PROPERTIES, ETC

All the 25 strains are motile and exhibit the poperty of ' swarming '   The organisms are on the average about 3μ in length and $0\,4\mu$ to $0\,5\mu$ in breadth and have rounded ends   Shorter forms and filamentous forms are very common   They are non-sporing

For fermentation tests the following carbohydrates were employed in 1 per cent concentration in peptone water along with Andrade's indicator—dextrose, galactose, maltose, lactose, saccharose, dulcite, mannite and salicin   Litmus milk was also included   Readings were made daily for a period of two weeks   Table I gives the fermentation reactions —

TABLE I.

Fermentation reactions, etc

A G = acid and gas in 24 hours
A = only acid in 24 hours
+ and — = presence or absence of particular reaction looked for
The number within brackets shows the day on which the reaction first appeared
O = Neither acid nor gas even after 14 days

Serial number	Strain	Dextrose	Maltose	Galactose	Lactose	Saccharose	Dulcite	Mannite	Salicin	Serum liquefaction	Indol
1	R.A.M.C	AG	AG	AG	O	AG	O	O	AG	—	+
2	National	AG	AG	AG	O	AG	O	O	AG	—	+
3	Shanmugam	AG	AG	AG	O	AG	O	O	AG	—	+
4	Kuala Lampur	AG	O	AG (3)	O	A (7)	O	O	A (14)	—	—
5	Kingsbury	AG	O	AG	O	AG (4)	O	O	A (14)	—	—
6	Manickam	AG	O	AG	O	AG (11)	O	O	AG (11)	+	—
7	4809	AG	AG	AG	O	AG	O	O	AG	—	+
8	Jayalakshmi	AG	AG	AG	O	AG	O	O	AG	—	+
9	Sastri	AG	O	AG	O	O	O	O	A (11)	+	—
10	Venkatachari	AG	AG	AG	O	AG	O	O	O	—	+
11	Pungavanam	AG	O	AG	O	AG (13)	O	O	O	+	—
12	Lily	AG	O	AG	O	A (10)	O	O	O	+	—
13	Bibi	AG	O	AG	O	O	O	O	O	+	—
14	Rokhia, Bee	AG	O	AG	O	AG (6)	O	O	O	+	—
15	Nagamma	AG	O	AG	O	AG (6)	O	O	O	+	—
16	Thomas	AG	O	AG	O	A (12)	O	O	O	+	—
17	Labrume	AG	O	AG	O	AG (6)	O	O	O	+	—
18	Brinkhurst	AG	O	AG	O	AG (6)	O	O	O	+	—
19	Jacob	AG	AG	AG	O	AG (8)	O	O	O	+	—
20	2270	AG	O	AG	O	AG (10)	O	O	O	+	—
21	Kanaka	AG	AG	AG (8)	O	AG	O	O	O	—	+
22	X19	AG	O	AG	O	A (9)	O	O	O	+	—
23	Anthonyswami	AG	O	AG	O	AG (11)	O	O	AG	—	+
24	Mrs, K	AG	O	AG	O	AG (12)	O	O	O	+	—
25	Tyagarajan	AG	O	AG	O	AG (12)	O	O	O	+	—

All the strains ferment dextrose readily with the production of acid and gas, but lactose, dulcite and mannite are not fermented   Galactose is fermented by all the strains but fermentation is late in the case of Kuala Lampur and X19   Eight strains, inclusive of the X19 strains, ferment maltose readily, there being no delayed fermentation of this carbohydrate   Only the maltose fermenting strains produce indol and none of them liquefies Lœffler's serum   All the maltose fermenters attack saccharose and salicin readily in 24 hours, with the exception of Venkatachari and 2270, which ferment saccharose alone   Strains which do not ferment maltose—the Kingsbury strains fall into this group—either do not ferment saccharose and salicin or fermentation is delayed, acid alone being sometimes formed   Serum is liquefied by 15 strains   None of these ferments maltose or produces indol   All the strains liquefy gelatin   In litmus milk there is usually an initial acidity within 24 hours followed by alkalinity and later decolorization of litmus and then peptonization With some strains peptonization was preceded by clotting

All the strains reduce nitrates to nitrites, are M R + and V P —   Hydrogen sulphide is produced by all and urea is decomposed with the production of ammonia Catalase and methylene blue reductase tests were positive in all

- Serology

Six agglutinating rabbit sera were prepared against 6 strains   Suspensions of 24-hour agar growths in saline were heated at 56°C for ½ hour and four graduated weekly injections were given intravenously to rabbits   A week after the last injection, the rabbits were bled by cardiac puncture

'H' suspensions were prepared by formalizing 24-hour broth cultures to a concentration of 0 4 per cent and suitably diluting them with formalized saline to yield an opacity of about 1 000 millions per c c   'O' suspensions were prepared by heating saline suspensions of 24-hour agar growths in the steamer for 1½ hours and diluting them to an opacity of about 1,500 millions, 0 5 per cent carbolic being added as a preservative

Agglutinations were carried out in Dreyer tubes in a water-bath at 55°C Readings were made at the end of 4 hours and again after 18 hours   There was no difficulty in differentiating flagellar from somatic agglutination   Dilutions ran in geometrical progression from 1 in 100 upwards

For absorption experiments the sera were diluted 1 in 25   Two c c of this transferred to a sterile centrifuge tube was mixed with 2 c c of a thick suspension in saline of the growth from 4 agar slopes of the appropriate strain   The mixture was incubated at 37°C for 4 hours and kept in the Frigidaire overnight   Controls in which saline was substituted for the suspension of agar growth were always included   The next morning the mixtures were spun down and the clear supernatant was used for agglutination with its homologous 'H' and 'O' suspensions

All the strains were tested for roughness by Millon's reagent (Bruce White, 1929) and several of them by the Thermo-agglutination test in normal saline   None was found to be rough

Table II gives the 'H and 'O' titres of the 6 sera against each of the strains in straight agglutination and also their titres against its homologous suspensions after absorption of agglutinins by the strains   The titres have been expressed in percentages

TABLE II

The upper row of figures against each strain represents the titre ('H' or 'O') expressed as a percentage of the homologous titre, the lower row represents, after absorption by that strain, the titre with homologous suspensions expressed as a percentage of the homologous titre of unabsorbed serum control

O = absence of agglutination in a dilution of 1 in 100

— = absorption not carried out

Serial number	Strain	Serum National 'H' 25,600	Serum National 'O' 6,400	Serum Kingsbury 'H' 3,200	Serum Kingsbury 'O' 400	Serum Sastri 'H' 3,200	Serum Sastri 'O' 400	Serum R A M C 'H' 3,200	Serum R A M C 'O' 400	Serum Pungavanam 'H' 12,800	Serum Pungavanam 'O' 1,600	Serum Rokha Bee 'H' 6,400	Serum Rokha Bee 'O' 400
1	R A M C	100	100	100	0	0	0	100	100	0	0	100	0
		0	0	25	100	—	—	0	0	—	—	100	100
2	National	100	100	0	0	0	0	60	100	0	0	0	0
		0	0	25	100	—	—	0	0	—	—	—	—
3	Shanmugam	100	100	100	0	0	0	30	100	0	0	0	0
		0	0	25	100	—	—	0	0	—	—	—	—
4	Kuala Lampur	100	0	100	100	0	0	200	0	25	0	100	0
		100	100	0	0	—	—	100	100	100	100	25	100
5	Kingsbury	100	0	100	100	0	0	200	0	25	0	100	0
		100	100	0	0	—	—	100	100	100	100	25	100

TABLE II—*contd*

Serial number	Strain	Serum National 'H' 25,600	Serum National 'O' 6,400	Serum Kingsbury 'H' 3,200	Serum Kingsbury 'O' 400	Serum Sastri 'H' 3,200	Serum Sastri 'O' 400	Serum R A M C 'H' 3,200	Serum R A M C 'O' 400	Serum Pungavanam 'H' 12,800	Serum Pungavanam 'O' 1,000	Serum Rokhu Bee 'H' 6,400	Serum Rokhu Bee 'O' 400
6	Manekun	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —
7	1800	100 / 0	100 / 0	100 / 18	0 / 100	0 / —	0 / —	100 / 0	100 / 0	0 / —	0 / —	100 / 50	0 / 100
8	Jayalakshmi	100 / 0	100 / 0	100 / 25	0 / 100	0 / —	0 / —	100 / 0	100 / 0	0 / —	0 / —	100 / 100	0 / 100
9	Sastri	0 / —	0 / —	6 / 100	0 / 100	100 / 0	100 / 0	0 / —	0 / —	100 / 0	100 / 0	0 / —	0 / —
10	Venkatachari	100 / 100	0 / 100	130 / 12	0 / 100	0 / —	0 / —	100 / 100	0 / 100	0 / —	0 / —	100 / 25	0 / 100
11	Pungavanam	0 / —	0 / —	0 / —	0 / —	100 / 0	100 / 0	0 / —	0 / —	100 / 0	100 / 0	0 / —	0 / —
12	Lily	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	0 / —	3 / —	0 / —	0 / —	0 / —

	13 Bibi	14 Rokhna Bee	15 Nagamma	16 Thomas	17 Labume	18 Brinkhurst	19 Jacob
	0 / 25	100 / 0	0 / —	0 / —	0 / —	0 / —	0 / —
	100 / 4	100 / 0	0 / —	0 / —	0 / —	0 / —	0 / —
	0 / —	0 / —	0 / —	0 / —	0 / —	75 / 0	0 / —
	0 / —	0 / —	0 / :	3 / —	1 / —	80 / 0	0 / —
	0 / 100	0 / 100	0 / —	0 / —	0 / —	0 / —	0 / —
	100 / 100	100 / 100	0 / —	0 / —	0 / —	0 / —	0 / —
	0 / —	0 / —	0 / —	0 / 100	0 / —	100 / 0	0 / —
	0 / —	0 / —	0 / —	25 / 100	0 / —	100 / 0	0 / —
	0 / —	0 / —	0 / —	0 / 100	0 / —	0 / —	0 / —
	0 / —	0 / —	0 / —	25 / 100	0 / —	0 / —	0 / —
	0 / 100	0 / 100	0 / —	0 / —	0 / —	0 / —	0 / —
	100 / 100	100 / 100	0 / —	0 / —	0 / —	0 / —	0 / —

TABLE II—concld

Serial number	Strain	Serum Vittorel H' 25,600	Serum Vittorel O 6,400	Serum Kingsbury H' 3,200	Serum Kingsbury O 400	Serum Sastri H' 3,200	Serum Sastri O 100	Serum R A M C H' 3,200	Serum R A M C O 400	Serum Tungavantu H' 12,800	Serum Tungavantu O 1,600	Serum Rolhr Leo H' 6,400	Serum Rolhr Leo O 100
20	2270	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —	0 —
21	Kuaeka	0 —	0 —	0 —	0 —	0 —	0 —	1 100	0 100	0 —	0 —	0 —	0 —
22	N19	100 0	100 0	0 27	0 100	0 100	0 100	70 0	100 0	0 —	0 —	0 —	0 —
23	Anthony s tne	0 —	0 —	0 —	0 —	12 100	0 100	0 —	0 —	2 100	0 100	0 —	0 —
24	Mrs K	0 —	0 —	7 100	0 100	100 0	200 0	0 —	0 —	40 0	100 0	0 —	0 —
25	Tyagarajm	0 —	0 —	0 —	0 —	100 0	0 100	0 —	0 —	100 0	0 100	0 —	0 —

It will be seen that strains National and R A M C are serologically identical as each absorbs the serum agglutinins of the other completely Six strains— R A M C, National, Shanmugam 4809 Jayalakshmi and X19—fall into one serological group Kuala Lampur, Kingsbury, Venkatachari, Bibi and Rokhia Bee give 'H' agglutination to full titre with these two sera but the 'H' agglutinins are not absorbed at all In the case of the last two strains there was distinct floccular agglutination with turbidity of supernatant in all dilutions up to full titre

Sastri and Pungavanam are also identical, as each absorbs out the 'H' and 'O' agglutinins of the serum of the other strain Sastri, Pungavanam, Brinkhurst and Mrs K fall into a second serological group

Similarly Kuala Lampur and Kingsbury fall into a third serological group

Strain Rokhia Bee stands alone No other strain gives 'O' agglutination with this serum, though several strains give 'H' agglutination and absorb the agglutinin to a certain extent

Seven strains do not agglutinate with any of the sera or agglutinate to a negligible titre

Previous workers have shown that while 'O' antigen has greater specificity 'H' antigen is more cosmopolitan and cannot be taken as a basis for serological classification This has been confirmed in the present study Strain Tyagarajan is agglutinated to full 'H' titre by serum Sastri (= Pungavanam) and absorbs its 'H' agglutinins completely With Kingsbury serum, 5 strains—R A M C, Shanmugam, 4809, Jayalakshmi and Venkatachari—give 'H' agglutination to full titre, but partially absorb the agglutinins Similarly, with serum Rokhia Bee, strains Kuala Lampur, Kingsbury, 4809, Venkatachari and Bibi give 'H' agglutination to full or very high titre but absorb the 'H' agglutinin incompletely

With serum National (= R A M C), strains Kuala Lampur, Kingsbury, Venkatachari, Bibi and Rokhia Bee give agglutination to full 'H' titre but do not at all absorb the 'H' agglutinin With serum Rokhia Bee, strains R A M C and Jayalakshmi behave similarly

A reference to Table II will show that several other strains give 'H' agglutination to a small fraction of the 'H' titre of the various sera

Regarding the X strains, Felix and Rhodes (loc cit) have shown that the aniridologenic strain Kingsbury is a variant of X19 and that whereas its 'H' antigen is in part identical with that common to all X strains, its 'O' antigen is completely different from that of both X2 and X19 strains From the present study it is seen that between the two Kingsbury strains and the two X19 strains (National and X19) there is no common 'O' antigen As regards the 'H' antigen however, the Kingsbury strains give 'H' agglutination to full titre with X19 serum without any absorption of the agglutinin With the Kingsbury serum there is no 'H' agglutination of the two X19 strains, but they partially absorb the agglutinin The strain R A M C however behaves differently From its biochemical and serological properties it appears to be an X strain It agglutinates with Kingsbury serum to full 'H' titre and partially absorbs the 'H' agglutinin

Though this aspect has not been sufficiently investigated, there is an indication that, as in the Salmonellas as shown by Andrewes (1922), organisms of the Proteus group are also probably biphasic,

Lastly, mention must be made of the three strains, Shanmugam, 4809 and Jayalakshmi   The history of their origin is not available, but from their names, they were evidently isolated locally   Both in their biochemical and serological reactions, they behave like X19 strains   Typhus fever being very uncommon in Madras, it is extremely unlikely that they were all isolated from typhus cases   As all known X19 strains originated from typhus cases and they stand by themselves in a serological group, it is very interesting that the three strains mentioned should behave in a manner similar to them

Summary

1   An examination has been made of the cultural, biochemical and serological reactions of 25 Proteus strains, most of which were isolated from fæces and urine and including X19 and Kingsbury strains obtained from other laboratories   Six agglutinating rabbit sera were prepared and the strains tested against them in straight agglutinations with ' H ' and ' O ' suspensions and with homologous suspensions after carrying out absorption tests with the different strains

2   All the strains gave ' swarming '.

3   The importance of maltose fermentation as a basis for classification has been confirmed   Eight strains, inclusive of X19 strains, which ferment maltose, also produce indol, do not liquefy serum, ferment saccharose readily, and with two exceptions ferment salicin also readily

4   Seven strains do not agglutinate with any of the sera or agglutinate to a negligible titre

5   There is no correlation between fermentation and serological reactions

6   By agglutination and absorption of ' H ' and ' O ' agglutinins, six strains, including X19 strains, fall into one serological group, four others into a second and the two Kingsbury strains into a third group

7   While the ' O ' antigen is more specific, there is a greater community of ' H ' antigen

8   X19 and Kingsbury strains are compared

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CULTIVATION OF VACCINIA VIRUS ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK-EMBRYO

BY

R SANJIVA RAO,

C G PANDIT,

AND

LIEUT -COLONEL H E SHORTT, I M S

(*From the King Institute of Preventive Medicine, Guindy*)

THE possibility of culturing the virus of vaccinia on the chorio-allantoic membrane of the chick-embryo was first demonstrated by Goodpasture and Woodruff (1931)

Since then other investigators, Stevenson and Butler (1933, 1934 and 1935), Lehmann (1934), Godhino (1934) and Sporozyski (1935), have confirmed and enlarged upon their findings

An appraisal of the work conducted so far by different workers suggests the possibility, as well as the desirability, of a large scale production of anti-smallpox vaccine by this method and its suitability for human vaccination has been considered after a thorough investigation of its immunizing, pathogenic and storage properties In our own work during the last two years we have attempted to study this problem in all its different aspects and to evaluate our experience, taking into consideration any limitations or drawbacks imposed on it by local conditions A communication based on the earlier part of our work has already been published (1934), and relevant portions have been reproduced here for the sake of completeness

TECHNIQUE

Goodpasture and Woodruff first used a neuro-testicular strain of the virus as the initial seed Stevenson and Butler (1933), preferring to use a purely dermal strain, initiated their work with a seed obtained by the inoculation of calf-lymph

into rabbits intradermally   This step ensured a bacteria-free condition of the seed-virus, while it retained its purely dermal character

Our own experiments were initiated by using clove-oil treated calf-lymph as the initial seed   This was rendered free from bacterial contamination by incubating several tubes at 37°C for 48 to 72 hours   A tube which proved sterile on bacteriological examination was then chosen   The contents were diluted 1 in 50 with normal saline and, after light centrifugalization, the supernatant fluid was used for inoculation   During the major portion of this work the technique described by Stevenson and Butler was followed with slight modifications   Latterly the improved technique described by Goodpasture and Buddingh (1935) in their very exhaustive communication on the subject has been adopted with very good results

Our actual procedure may be described as follows —

Fertile eggs 14 to 15 days old incubated in a special egg incubator at 39°C are selected   Stevenson and Butler (1933) used 11 days old eggs, but we have obtained better results with those 14 to 15 days old as, in these, the more advanced development of the embryos ensures a better exposure of the chorio-allantoic membrane to the virus   Previous to inoculation, the eggs are candled inside a small wooden frame lined with black cloth   All doubtful eggs are discarded, and the good ones returned to the incubator

To ensure sterility of operation, the inoculation of eggs is carried out inside a glass chamber   This is cleaned daily on the inside with an antiseptic before the operation   For cutting an opening through the egg-shell, carborundum discs, worked by an electric motor through a flexible shaft, are used   The site of inoculation is first thoroughly cleaned with 5 per cent carbolic followed by absolute alcohol , a triangular opening, with sides about half to three-quarters of an inch each, is next made in the egg-shell   The size of the window does not influence materially the size of the lesions and can be varied with the size of the egg   The disc is driven at the lowest possible speed to avoid injury to the shell membrane , a little practice enables one to feel when the disc has just cut down to the shell membrane and a change in the note produced during cutting is apparent at the same time   The area outlined by the three cuts is now covered over with a layer of melted paraffin at about 56°C   The egg is then immediately placed over a wooden egg-holder inside the inoculating chamber and, with the point of a sharp sterile cataract knife, the shell membrane is cut obliquely—to avoid injury to the chorio-allantoic membrane —along two sides, leaving the third side to act as a hinge to the flap cut   The coat of paraffin helps to keep the piece of shell intact   The flap is then raised and the chorio-allantoic membrane, thus exposed, is inoculated with the material   The flap is then closed, the cut edges are covered over with melted paraffin, and the egg marked and returned to the incubator with the cut side up   The inoculated egg is opened on the third day for collecting the lesions   Here, our findings confirm the observations of Goodpasture and Buddingh (*loc cit*) that harvesting the virus on the third day, instead of on the fourth, gives better results   On the fourth day there is more necrotic tissue and, probably, less virus, as our experience has shown that the potency of the lesions does not correspond with their size

The egg is placed in the holder with the pointed end upwards   The upper portion is cleaned with 5 per cent carbolic, and then with absolute alcohol and flamed   The top is then cut open and the embryo extracted   The chorio-allantoic

membrane, which remains slightly adherent to the shell membrane, is pulled out with sterile forceps, placed in a sterile Petri dish and covered with 50 per cent glycerine water The lesion is then cut out and stored in 50 per cent glycerine water in a refrigerator The whole procedure is carried out inside the glass chamber

We have adopted the method of grinding lesions from each membrane separately, or in batches of two each, when there is a large number, and then testing for sterility This ensures against loss of the bulk of the material due to contamination from any one of the lesions The grinding is conducted inside a glass chamber washed previously on the inside with an antiseptic The lesions are ground in a sterile glass mortar with a little glass sand, and 50 per cent glycerine water is added in the proportion of 1 4 and the suspension then stored in the refrigerator This forms our 'undiluted virus', and is used for the next serial passage For this purpose, we at present dilute the virus with an equal volume of sterile distilled water

Since the adoption of our present technique, good lesions are the rule, although, as we have already observed, the size of lesions usually does not go hand in hand with titrable potency

At each passage, lesions have not been pooled as we thought such a step might mask any alteration in the character of the virus should it occur The titrations have been carried out on calves as a matter of convenience, for a large number of samples can be tested in different dilutions on one calf

Two calves have been used for each test to eliminate errors arising from refractoriness in a single calf The usual scarification technique was employed For each dilution an area about $\frac{3}{4}$ inch square was lightly scarified and about 0 02 c c of the membrane virus was spread over the respective areas

The results are summarized in Table I —

TABLE I

Passage number	Highest titre	Passage number	Highest titre	Passage number	Highest titre
1	1 100	10	Undiluted	19	1–100
2	1–200	11	1–25	20	1–100
3	1–500	12	1–10	21	1–100
4	Undiluted	13	1–10	22	1–100
5	,,	14	1–10	23	1–100
6	,,	15	1–10	24	1–100
7	1–25	16	1–10	25	1–500
8	1–100	17	1–100	26	1–100
9	Failure	18	1–100	27	1–50

TABLE I—*concld*

Passage number	Highest titre	Passage number	Highest titre	Passage number	Highest titre
28	1–10	43	1–10	58	1–50
29	1–500	44	1–50	59	1 50
30	1 500	45	Undiluted	60	1–100
31	1–500	46	1–50	61	1-100
32	1–1,000	47	1–25	62	1–100
33	1–50	48	1–25	63	1–200
34	1–500	49	1–25	64	1–100
35	1–250	50	Undiluted	65	1–100
36	1–500	51	1–10	66	1–250
37	1–10	52	Undiluted	67	1–500
38	Undiluted (?)	53	1–50	68	1 500
39	,	54	1–50	69	1–1,000
40	1–10	55	1–50	70	1–1,000
41	Undiluted	56	Undiluted		
42	1–10	57	1–100		

From the above table it will be seen that at any stage the potency of the membrane virus never reached the high standard of this laboratory calf-lymph, which never gives a titre of below 1 in 1,500, while usually the titre is above 1 in 8,000

We have to note in this connection that almost all other workers on this subject used rabbits for the test Our experience is that rabbits are considerably more susceptible to the virus than calves, and so may be expected to show a higher titre The comparatively low titres may partially be explained on this ground The potency, which shot up to 1 in 500 in the third passage, suddenly, for no accountable reason, dropped down to undiluted and continued to maintain a low level till the 16th passage, when it again rose to a general level of 1 in 100 and, with one or two minor fluctuations went up to 1 in 1,000 A considerable drop is again noticed and it is curious to note a very low titre in the 39th and 40th passages when the membrane lesions were characteristically big In the 47th passage ducks' eggs were interposed with a view to investigate whether a change in the medium for culture would enhance the virulence of the virus It is common knowledge that the virulence of seed-virus used in the manufacture of calf-lymph is maintained at a high level by successive passages through calf and rabbit, and in our own

laboratory a modified Nijland cycle has been adopted, viz, calf-rabbit-buffalo[1]-buffalo[2]-calf

On these grounds, although contrary to the experience of Goodpasture and Buddingh, who observed no drop in potency throughout the very large number of passages they have made, it is reasonable to suppose that the virus cannot maintain the same high level of infectivity without a change of host   Working on the cultivation of vaccinia virus in tissue culture by the technique of Rivers, Coffey (1934) and Chen (1934) observed that the titrable potency gradually dropped, and the latter author enhanced the virulence of the culture by the interposition of rabbit testicular tissue in place of chick-embryonic tissue

The results of our experiments with ducks' eggs are, as the table shows, not at all conclusive   Yet, in our opinion, the potentialities of this step in the manufacture of membrane virus require to be investigated

STORAGE QUALITIES

The keeping qualities of the membrane virus were investigated in comparison with those of calf-lymph   A sample of calf-lymph on the basis of preliminary titration was diluted down to 1 in 1,500 to approximate its potency to that of a sample of membrane virus used in the test   Each virus was divided into three lots, and these three lots were distributed in pairs at different temperatures   The first was kept at 3°C, the second at 10°C, and the third at room temperature, which averaged between 30°C and 35°C   The samples were titrated on calves at the end of every week

The results are summarized in Table II —

TABLE II

Nature of virus	Temperature	HIGHEST TITRE AT END OF			
		1 week	2 weeks	3 weeks	4 weeks
Membrane virus P V 2 Titre 1–100	R T	Nil	Nil	Nil	Nil
	10°C	1–10	Undiluted	Nil	Nil
	3°C	1–10	1–10	1–10	Nil
Lymph 149 diluted to titre 1 100	R T	Nil	Nil	Nil	Nil
	10°C	1–50	1–10	1–10	Nil
	3°C	1–100	1–100	1–100	1–10

As will be seen from the above table, the keeping qualities at 3°C though not so good as those of the considerably diluted calf-lymph are quite commensurate with the initial low titre of the sample   We are inclined to attribute the poor results compared with those obtained by Goodpasture and Buddingh, firstly, to the initial

low titre of the sample and secondly to the fact that the calf was used for titration a reference to which has already been made   Our results however, regarding the rapidity of deterioration at higher temperatures   more or less corroborate their findings

IMMUNIZING PROPERTIES OF THE MEMBRANE VIRUS

Experiments were made to study the immunizing properties and the nature of the reactions produced by membrane virus in comparison with those of calf-lymph Three monkeys (*M simicus*) were vaccinated, No 1 with membrane virus alone, No 2 with calf-lymph alone and No 3 with both membrane virus and calf-lymph The membrane virus was used undiluted and the calf-lymph in a dilution of 1 in 25 to approximate their titres as far as possible   0 03 c c of inoculum was spread and rubbed over a scarified area $\frac{3}{4}$ inch square, and daily observations regarding temperature and reaction were made

The results are given in Table III —

TABLE III

Animal	Virus inoculated	Nature of reaction
Monkey I	Membrane virus	No reaction at 48 hours marked elevation of scarified area at 72 hours Confluent vesiculation at 96 hours Reaction then gradually subsided and scab fell off on the 20th day, leaving a good scar Temperature reaction identical with that of monkey II
Monkey II	Calf lymph	Early reaction was noted and there was evidence of a good take at 48 hours Passed through the same stages of development as monkey I and the scab fell off on about the same day Usual temperature reaction
Monkey III	Both membrane virus and calf lymph	Both inoculations showed identical stages of development as were noted in monkeys I and II respectively namely early reaction with calf lymph and delayed reaction with membrane virus Accidental vesicles were noted on the 5th day in the case of membrane virus, probably attributable to the movements of the leather belt with which the monkey was tied

Viricidal tests of the sera of the monkeys were then   conducted with a view to gauging the extent of immunity produced by membrane virus as compared with that produced by calf-lymph

The monkeys were bled on the 13th day after vaccination and the sera obtained were tested against both the membrane virus and calf-lymph, the latter being diluted 1 in 50 to equalize its titre to that of membrane virus   Each serum was tested in 4 dilutions, viz , undiluted, 1 in 5, 1 in 10 and 1 in 20   0 2 c c of each

virus was mixed with 0 2 c c of each dilution of the two sera, the mixtures incubated at 36°C for two hours and then 0 03 c c of each was tested on two calves

Results are summarized in Table IV —

TABLE IV

Final serum dilutions	Membrane virus			Calf lymph		
	Serum I	Serum II	Normal monkey serum	Serum I	Serum II	Normal monkey serum
1–2		3	2			1
1–10		4	6		2	Confluent take
1–20			3	1	3	7
1–40	1		5	?	6	7

Further experiments were done on rabbits Two batches of rabbits were vaccinated, one with membrane virus and the other with calf-lymph diluted 1 in 1,000   One lot in each batch was re-vaccinated with routine calf-lymph at the end of 6 weeks, and another at the end of 9 weeks   All the re-vaccinated rabbits failed to take

These experiments on monkeys and rabbits bring out two important facts Firstly, that there is no difference between membrane virus and calf-lymph as regards the nature of the reaction, other than that in the case of membrane virus, the reaction appears 24 hours later than in the case of calf-lymph secondly, that no appreciable difference in the immunizing power of the two viruses is demonstrable

ALTERATION IN THE CHARACTER OF THE VIRUS

Experiments have been carried out to discover if any change in the direction of neurotropism has taken place in the truly dermal nature of the virus as a result of serial passages in eggs Two batches of rabbits were inoculated one with 1 c c intra-nasally and the other with 0 1 c c sub-durally, with each virus The rabbits were sacrificed at the end of 1 week, 2 weeks and 3 weeks and their brains tested on calves Neither series of rabbits showed any evidence of the presence of the respective viruses in their brains A few of the rabbits were kept under observation for over 10 weeks and none of them developed any symptoms of encephalitis

CONCLUSIONS

1 A review of the immunizing and pathogenic properties of membrane virus fully corroborates the observations of other workers on its potentialities for human prophylaxis against smallpox, but it has to be remembered that its immunizing

value has only been tested against *vaccinia* and not against smallpox as it occurs under natural conditions

2   We consider that its storage qualities and average potency, while not yet equal to those of calf-lymph, may be considerably improved by better technique

3   Although membrane virus does not come up to the level of calf-lymph in its standard of potency we have no reasons to believe that the immunity produced by such a virus will necessarily be less

4   Cultivation of vaccinia virus on the chorio-allantoic membrane of chicks has all the simplicity of technique and cheapness in cost to be desired

5   The one pre-requisite to manufacture on a large scale is an unfailing supply of fertile eggs, a condition not easily attainable in Madras during several months in the year

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CULTIVATION OF THE VIRUSES OF SANDFLY FEVER AND DENGUE FEVER ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK-EMBRYO

BY

LIEUT.-COLONEL H E SHORTT, I M S ,

R SANJIVA RAO,

AND

C S SWAMINATH

(From the King Institute of Preventive Medicine, Guindy, Madras)

SHORTT, POOLE and STEPHENS (1934, 1935) have given an account of preliminary experimental work on the virus of sandfly fever and the present account is an extension of that work

The successful cultivation of various viruses by the original method of Woodruff and Goodpasture (1931) on the chorio-allantoic membrane of the embryo-chick led us to apply this method to blood and sera from cases of sandfly fever and dengue

MATERIALS AND TECHNIQUE

The sandfly fever sera were sent to us from the Military District Laboratory, Peshawar, and consisted of fresh liquid sera, old liquid sera and dried sera

The dengue sera were obtained locally in Madras during an epidemic of this disease In this case all the sera used were fresh, while, in addition, in some cases citrated whole blood was used

The full technique of inoculation of eggs with virus material of any sort has been described by us in another communication (Rao, Pandit and Shortt, 1936) and need not be repeated here

SANDFLY FEVER

The eggs inoculated with serum from cases of sandfly fever were examined after intervals of 4 days

In Table I are given the results of all the sera tested —

TABLE I

Experiments with sera of sandfly fever cases

Serum number	Number of passages															
	1		2		3		4		5		6		7		8	
	N I	N P	N I	N P	N I	N P	N I	N P	N I	N P	N I	N P	N I	N P	N I	N P
Pooled fresh liquid sera from 4 cases	4	,	2	2	1 8	0										
Dried serum 7	2	0														
Dried serum 8	2	2	5	2	1	2	2	1	2	1	1	1	1	1	2	1

Eggs found putrid on opening have not been taken into account

F = Filtered through collodion membrane (A P D 0 9μ) on account of contamination

N I = Number of eggs inoculated

N P = Number of eggs positive

In reading this table, it must be emphasized that it is highly probable that a considerable proportion of the sera tested had no viable virus   They were sent by post from Peshawar to Madras and, in most cases, had already been stored for considerable periods in Peshawar before despatch   It will be seen that subcultures have been obtained up to eight passages to date

LESIONS PRODUCED BY SANDFLY FEVER VIRUS

The lesions on the chorio-allantoic membrane were usually examined on the 4th day after inoculation   In positive preparations there was a distinct thickening of the membrane at the centre of the lesion producing a dense opaque patch   The area of these lesions varied but some measured as much as 10 mm in diameter   Surrounding the central lesion for a variable distance, there was a definite opacity of the chorio-allantoic membrane which might cover an area of 1 5 cm in diameter, including the central lesion (Plate XXIX, fig 1)   The lesions were fixed in Sansom's modification of Carnoy s fluid, double embedded in celloidin and paraffin and sections cut at right angles to the surface   Sections were stained by Giemsa's, haemalum and Mann's stains

The sections stained by haemalum naturally gave the best idea of the cellular changes involved in the lesion

In the following description, for the sake of brevity, we do not propose to go into the various complications of folding of the membranes on one another to form the chorio-allantoic membrane and will limit our terms to three. Although this may not be a strictly correct procedure from the zoologist's point of view it will greatly facilitate our description. The three terms we propose to use are entoderm, for the layer of cells on the inner aspect of the chorio-allantoic membrane; ectoderm, for that on the outer aspect; and mesoderm, for the space contained between the two layers. It was noted that both the ectoderm and entoderm, especially the latter, were bulged out over the area occupied by the lesion in order to accommodate an intense cellular infiltration in the mesoderm (Plate XXIX, fig 2). The ectoderm over the most prominent part of the lesion was absent and presumably had necrosed in a manner similar to the formation of a minute ulcer (Plate XXIX, fig 2). The entoderm, which normally shows a thickness of two layers of cells, exhibits marked proliferation and is many cells thick over the lesion (Plate XXIX, fig 3). This proliferation is most marked over the centre of the lesion and grades off towards its periphery so that the transition to the normal two-cell thickness is a gradual one. Between the ectodermal and entodermal layers of the normal chorio-allantoic membrane the general arrangement of the cells of the mesoderm is parallel to the surfaces and they are normally comparatively sparsely distributed. In the lesion under consideration, apart from the necrosis noted above, the ectodermal and entodermal epithelial layers contain a wide space of mesoderm closely packed with cells in a fibrous-looking stroma.

The origin of these cells is a matter of interest and importance. A careful study of the mesoderm will reveal the fact that the majority of the cells have the appearance of epithelium and, if they are epithelium, they must have come from either the ectodermal or entodermal layers. The entoderm, although greatly hypertrophied, as already described, is intact over the lesion and has a definite and well-marked limit internally, i.e., it shows no evidence of extension into the mesoderm (Plate XXIX, fig 3). It can, therefore, be ruled out as the origin of the infiltrating cells.

In the case of the ectoderm, on the other hand, the proliferation of cells seen is not limited internally and the inner cells appear definitely to infiltrate the mesoderm. Columns of them may be seen growing downwards into the latter and it is possible that the majority of the cells seen in the mesoderm have this origin. The appearances seen strongly resemble the infiltration of deeper tissues by an epithelium of surface origin and this, in essence, is what the lesion appears to be from the histological point of view. While giving this description of the origin of the infiltrating cells we draw attention to our statement that our definitions of ectoderm and entoderm may not be zoologically accurate and we are prepared to find that the infiltrating cells are actually derived from the layer of cells lying immediately *under* the ectoderm and, therefore, presumably of mesodermal origin— in most probability fibroblasts.

INCLUSION MATERIAL

In sections stained by Giemsa's stain or Mann's stain there is found to be a condition of massive growth of inclusion material in the cells of the mesoderm. We have not used the term inclusion 'bodies' for the reason that in many cases

the whole cytoplasm of the cells appears to be replaced by red-staining inclusion material This material is sufficiently massive, in many cases, partially to mask even the nucleus of the containing cell The extent of this inclusion material may be seen in the illustration (Plate XXIX, fig 4) representing a part of the lesion

The nuclei of some of the cells appear to show oxychromatic degeneration but, in the presence of many other oxychromatic dots in the section, we are not prepared especially to emphasize this point

DENGUE FEVER

The blood of dengue fever cases was taken generally on the first day of fever The results of cultures and other information are detailed in Table II —

TABLE II

Giving detailed information about the dengue fever cases

Number of case	Date of onset of fever	Date of taking blood	Clinical diagnosis	Material inoculated	Result of inoculation on the chorio allantoic membrane
3	8–10–35	8–10–35	Dengue	Serum	Positive
4	8–10–35	8–10–35	Cold	,	Negative
5	11–10–35	11–10–35	Not dengue	,	,
6	10–10–35	10–10–35	Dengue	,,	Positive
7	10–10–35	10–10–35	,	,	,
8	14–10–35	14–10–35	,	,	Negative
10	17–10–35	18–10–35	,	,,	Positive
11	17–10–35	18–10–35	,	,	,
12	20–10–35	20–10–35	,	,,	,
14	22–10–35	22–10–35		Plasma	,
17	26–10–35	26–10–35	,	,,	Negative
18	28–10–35	28–10–35		,,	Positive
19	28–10–35	28–10–35		,,	,,
20	28–10–35	28–10–35		,,	,,
21–26 (pooled)	28–10–35	28–10–35		Serum	,,
27–31 (pooled)	30–10–35	30–10–35		,	,,
39	10–11–35	10–11–35		Plasma	Negative
40	11–11–35	11–11–35	,	,	,,
42	12–11–35	12–11–35			Positive
43	18–11–35	18–11–35	,	,	,,
44	22–11–35	25–11–35			,,

It will be seen from the table that out of 19 cases with a clinical diagnosis of dengue 15 cases gave a positive culture on the chorio-allantoic membrane

PLATE XXIX
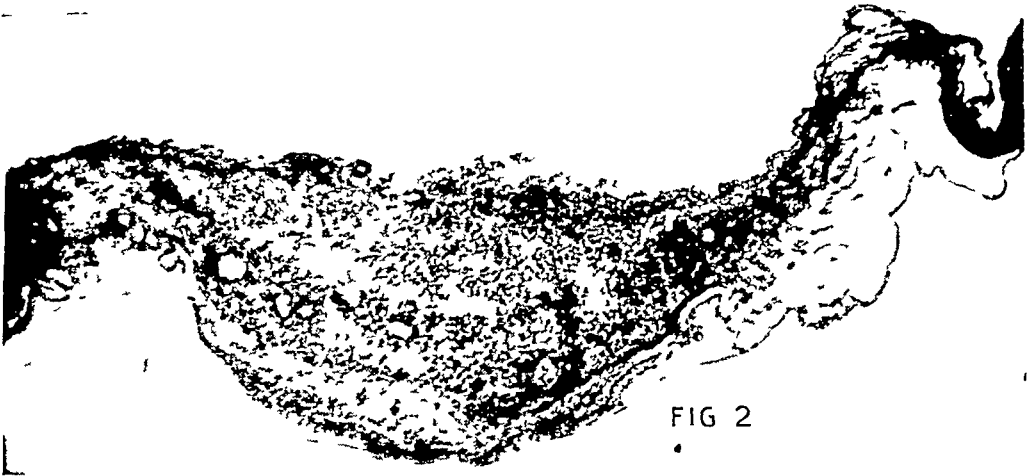
FIG 2
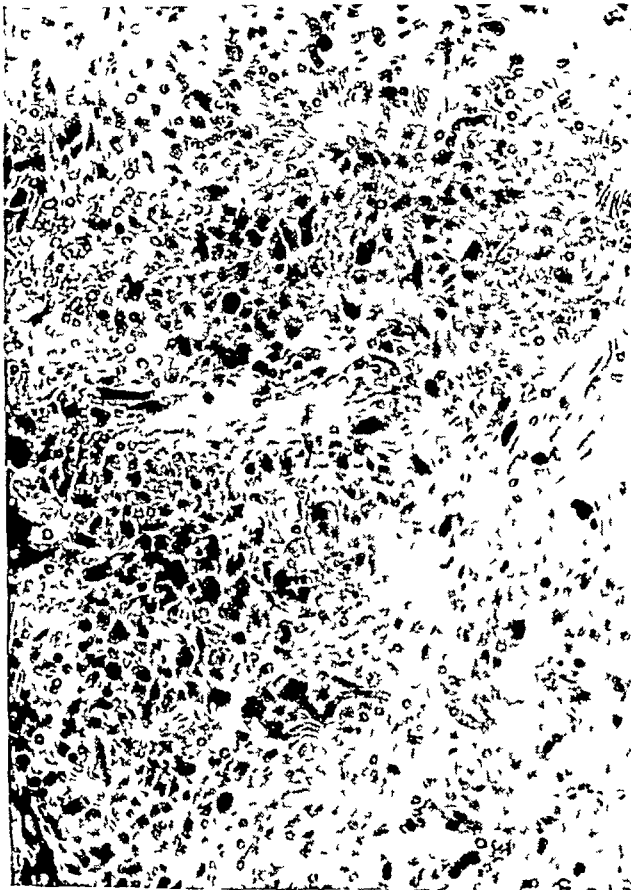
FIG 4

FIG 1

FIG 3

Fig 1   Naked eye appearance of sandfly fever lesion
  „   2   Low power appearance of sandfly fever lesion
  „   3   Hypertrophied but intact entoderm
  „   4   Inclusion material in cells of mesoderm in sandfly fever lesion

PLATE XXX

FIG 6
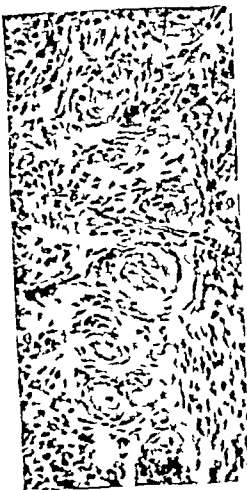
FIG 7

FIG 5

Fig 5   Naked eye appearance of dengue fever lesion
  ,,   6   Lesion of dengue fever
  ,,   7   'Cell nests' in of lesion dengue fever

Fuller details of cultures of the dengue fever cases are given in Table III —

TABLE III

Experiments with fresh sera from cases diagnosed as dengue fever

Serum number	NUMBER OF PASSAGES											
	1		2		3		4		5		6	
	N I	N P	N I	N P	N I	N P	N I	N P	N I	N P	N I	N P
3	2	2	Contaminated									
6	2	2*										
7	2	2*										
8	3	0										
10	2	2	2	2	1	0						
11	2	2	Contaminated									
12	1	1	(Fixed in Bles and photographed)									
14	6	4	3	2	3	2	1	1	1	1		
17	2	0										
18	2	2	Putrid eggs									
19-20	4	2	1	0								
21-26	2	2	1	1	Putrid eggs							
(Filtered) 21-26	2	2										
27-34	1	1	1	1	1	1						
39	2	0										
40	1	0										
42	2	2	4	2								
13	1	1										

* Lesions were not marked and so were not passaged
N I = Number inoculated
N P = Number positive
Eggs found putrid on opening have not been taken into account

It will be seen from the table that subcultures have been obtained up to five passages to date, also that, in one case, lesions were obtained with serum filtered through a collodion membrane of average pore diameter 0.8μ

LESIONS PRODUCED BY DENGUE FEVER VIRUS

The lesion in dengue fever (Plate XXX, fig 5) differs to some extent from that in sandfly fever   Macroscopically it shows as an opaque area on the fresh membrane but appears to be flatter so that, in section, it shows as a thickened area with roughly parallel sides instead of with curved bulging sides as in the sandfly fever lesion

Microscopically there are also points of difference

We see the same thickened area of the chorio-allantoic membrane with hypertrophied ectoderm and entoderm, although this is less marked than in the case of sandfly fever   The entoderm shows a rugose type of hypertrophy (Plate XXX, fig 6) and this hypertrophy is seen in the same figure to be most marked at the periphery of the lesion and practically to disappear at its centre

The ectoderm is also hypertrophied but less so and there is the same evident invasion of the mesoderm as was described for sandfly fever   The mesoderm is infiltrated by cells which have a general longitudinal direction under both ectoderm and entoderm but are more irregularly arranged in the centre (Plate XXX, fig 6) The origin of these cells is uncertain but they appear very similar to those in the lesion of sandfly fever and are almost certainly similar in origin   In certain situations formations are seen with the closest resemblance to the 'cell nests' seen in certain types of epithelioma (Plate XXX, fig 7) and inevitably call to mind the viral theory of the causation of epitheliomas

INCLUSION MATERIAL

This is apparently distributed exactly as in the sandfly fever lesions and is equally massive in character

DISCUSSION

The lesions described as occurring in the chorio-allantoic membrane of the chick as the result of inoculation with the viruses of sandfly fever and dengue fever are not, from the histological point of view, especially characteristic, as very similar lesions may be produced with other agents   The important point appears to be that the lesions are produced with great regularity, can be passaged in series and are not obtained in controls with normal serum   The presumption, therefore, is that they are really caused by the viruses of the two fevers under consideration

Should this prove to be the case the question of the practical application of this technique from the point of view of preparation of vaccines and sera against these diseases will have to be considered and sandfly fever, at least, is a disease sufficiently important in the army in India to make it worth the while of the Military medical authorities to follow up the line of investigation indicated by the results we have recorded above

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FILARIASIS IN PATNAGARH (ORISSA FEUDATORY STATE)

BY

S SUNDAR RAO

(*From the Filariasis Research Laboratory, Calcutta School of Tropical Medicine*)

PATNAGARH is a town in Patna State in Orissa and, till 1899, was the capital of Patna State   It is situated in 20°43 N and 83°10′E , at an elevation of 500 ft above sea-level   The country around is very uneven with hill ranges rising in various directions covered by jungle and interspersed with rivers   The soil is fertile being for the most part light and sandy

The area of Patnagarh proper is about one square mile   Patnagarh is more rural in character than urban   There are many large tanks in the town

Patnagarh has long been known to be intensely infected with filariasis   A survey of the town was carried out by the author at the instance of the State authorities and the results of the survey are reported in this paper

Several interesting points were recorded during the survey, which was carried out in February 1933   For instance, it was observed that filarial infection occurring in the human population in Patnagarh is not due to *Wuchereria bancrofti*, the common parasite occurring in most parts of India, but to the worm provisionally known as '*Filaria*' *malayi* Brug, of which only the microfilaria is yet known, this being entirely different from the microfilaria of *Wuchereria bancrofti*, as are also the pathological effects produced by it

The area that has been surveyed is shown on the Map   The town of Patnagarh has a population of 3,887 and is the second biggest in the State   The population comprises different classes of people, chiefly Oriyas, and includes a large proportion of semi-aboriginal people—the Binjhals, Saharas, Konds, Gandas, Ghasis and Koles, Hindus in religion   Agriculture is the chief occupation

Water supply is obtained from wells, tanks and the river Subarnarekha   There are no drains in the town, nor conservancy, except hand-removal of night-soil from a few latrines attached to the state buildings and schools   The rest of the population use scrub jungle or open ground for defæcation

MAP OF PATNAGARH

Scale 5 inches = 1 mile

(Structures shown in Wards 2, 3 and 7 indicate temples)

Number	Ward	Houses	Population
1	Brahmanpara	92	410
2	Budapara	153	705
3	Jadbandpara	52	173
4	Gadbhitar	46	205
5	Kolpara	73	285
6	Ampara	37	125
7	Bhulnapara	135	769
8	Baharpara	114	583
9	Kadopara	75	326
10	Tikapalli	27	98
11	Ghasipara	22	88
12	Gandapara	28	120

River Subarna Rekha

River Subarna Rekha

Gha Band

Vosagar

Hira Sagar

Ram Sagar

Venu Sagar

Asram Sagar
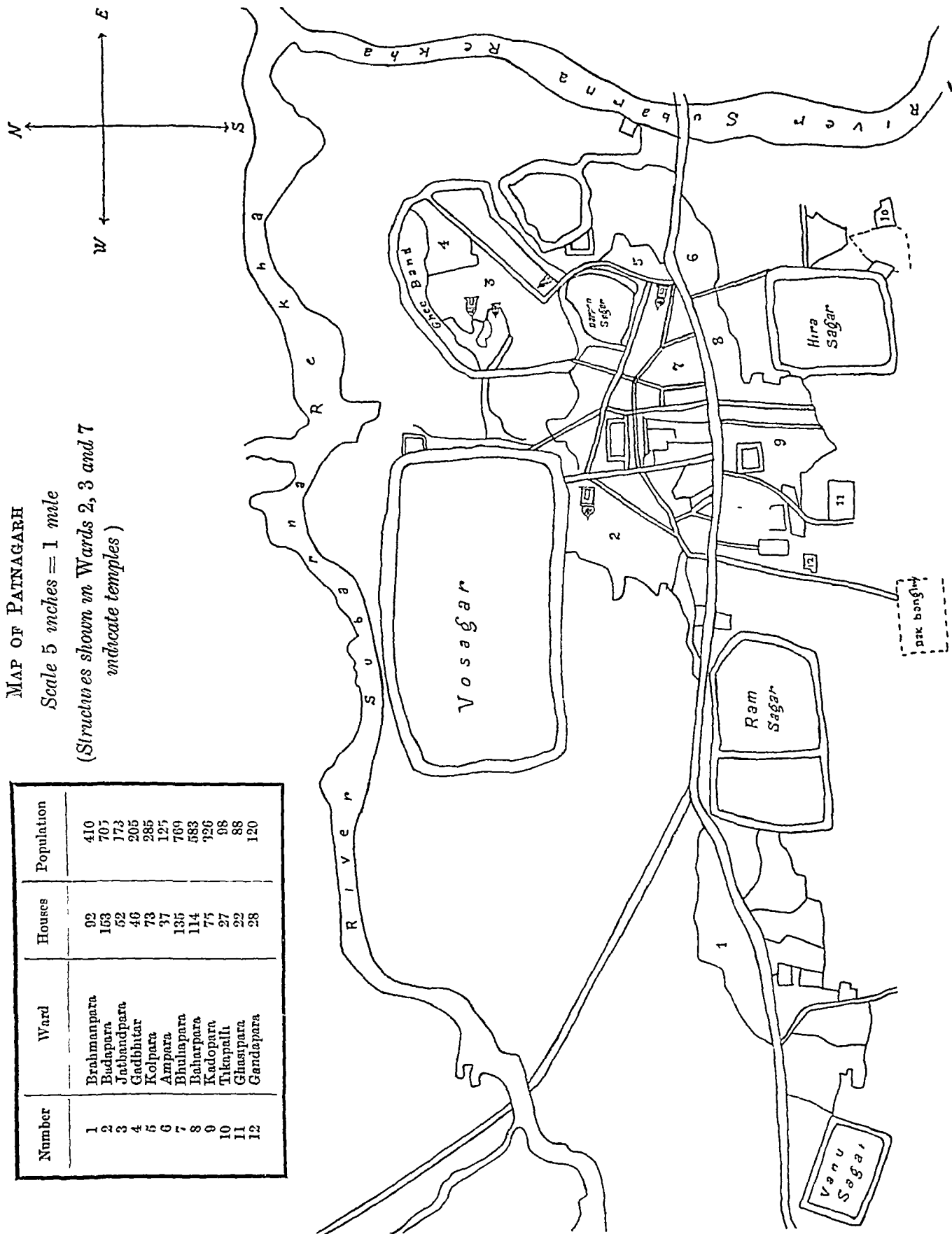
The climate is similar to that prevailing in the rest of Orissa except that it is probably hotter in summer and colder in winter   The average annual rainfall is about 50 inches and is received chiefly during the south west monsoon, from July to September   The prevailing diseases in the order of frequency are malaria, skin diseases, dysentery, urethritis, hookworm infection and leprosy

Patnagarh is divided into 12 wards, the wards generally being named according to the caste of the people residing in the area   Ward 1, Brahmanpara, inhabited by Brahmins, is situated on the west and is isolated from the rest of the wards The other wards lie close to one another and form the major portion of the town The Map also shows the wards into which the municipal area is divided and the total number of houses and population ward by ward in Patnagarh

Filarial infection in Patnagarh

To determine the incidence of filarial infection in the different wards of Patnagarh the people were examined at night between 10 p m and midnight, and thick smears of peripheral blood from the finger were taken for subsequent examination A total of 1,047 persons were examined, representing 37 per cent of the population which is a very good percentage for random sampling   The results have been tabulated (Table I), showing the details in regard to the microfilaria rate and the filarial disease rate of the 12 wards of Patnagarh

(a) *Microfilaria rate of Patnagarh* —The microfilaria rate of Patnagarh works out at 17 8 per cent (186 persons showing microfilariæ out of 1,047 examined) When the different wards of the town are considered separately marked variations in the intensity of the infection in different wards are observed   Ward No 4 (Gadbhitar) is the most heavily infected ward, the microfilaria rate being 40 0 per cent   The more heavily infected wards in the order of infection intensity are Gadbhitar, Ghasipara, Tikapalli, Ampara, Brahmanpara, Kadopara, Jatbandpara, Gandapara, Kolpara and Badapara, while wards with light infection are Bhuliapara and Baharpara   The first ten ' paras ' lie on the outskirts of the town and are situated in the immediate neighbourhood of the tanks, while the least infected ones (4 5 per cent and 3 2 per cent) are centrally situated

Table II shows the total number of persons harbouring microfilariæ in the blood at different ages   In the first group, ages 1 to 10, there were 38 children below the age of 5, of these 24 were male and 14 female   Six among the former and two in the latter were positive for microfilariæ   The youngest child showing the infection was aged 4

From the above table it appears that the percentage of infection is practically the same for all the age periods   The results show a striking difference from those usually obtained with *Wuchereria bancrofti* infection   In the latter case the infection curve shows a rapid increase during the age period of 10 to 20 or 30 and thereafter a gradual but steady increase   As regards the distribution in the sexes, however, no marked difference is noticeable in the case of either of these infections

(b) *Filarial disease rate* —A house to house survey for filarial diseases was carried out and the results are shown in Table I   Out of a total number of 3,887 persons examined during this survey 127 were found to have filarial diseases or

gave a history of filarial lymphangitis   The gross filarial disease rate for Patnagarh is 3 3 per cent   Here again the filarial disease rate is highest in the wards lying on the boundary of the town, such as Gandapara and Gadbhitar, while Baharpara and Bhuliapara show the lowest rates   It will be remarked that there is a general positive correlation between the microfilaria rate and the filarial disease rate, and that areas with heavy infection have a high incidence of filarial disease and vice versa

TABLE I

Showing the filarial disease rate and the microfilaria rate of Patnagarh ward by ward

Serial number and names of wards	FILARIAL DISEASE RATE			MICROFILARIA RATE		
	Total population (all examined)	Number with signs of filarial disease,	Filarial disease rate, per cent.	Number examined for microfilaria	Number positive for microfilaria	Microfilaria rate, per cent
1 Brahmanpara	410	25	6 1	96	25	26 0
2. Badapara	705	21	3 0	163	19	11 7
3 Jatbandpara	173	3	1 7	54	13	24 0
4 Gadbhitar	205	20	9 8	55	22	40·0
5 Kolpara	285	5	1 8	63	9	14 3
6 Ampara	125	9	7 2	26	7	27·0
7 Bhuliapara	769	4	0 5	205	9	4 5
8 Baharpara	583	5	0 9	125	4	3 2
9 Kadopara	326	9	2 8	88	22	25 0
10 Tikapalli	98	5	5 1	42	15	35 7
11 Ghasipara	88	8	9 1	88	32	36 4
12 Gandapara	120	13	10 8	42	9	21 4
TOTALS	3,887	127	3 3	1,017	186	17 8

Table II

Showing the microfilaria rate according to age and sex

Serial number	Age group	MALE		FEMALE		TOTALS		PERCENTAGE
		Total examined	Number showing micro filariæ	Total examined	Number showing micro filariæ	Number examined	Number showing micro filariæ	
1	1–5	24	6	14	2	38	8	21 0
2	6–10	94	13	73	12	167	25	15 0
3	11–15	90	14	10	1	100	15	15 0
4	16–20	87	10	13	6	100	16	16 0
5	21–25	72	9	8	3	80	12	15 0
6	26–30	157	30	20	7	177	37	20 9
7	31–35	97	20	19	2	116	22	19 0
8	36–40	65	13	20	2	85	15	17 6
9	41–45	37	8	9	2	46	10	21 7
10	46–50	53	9	10	5	63	14	22 2
11	Over 50	63	9	12	3	75	12	16 0
	TOTALS	839	141	208	45	1,017	186	17 8

Table III

Showing the types and incidence of filarial diseases

Filarial disease (Total cases 127)	Male	Female	Totals	Percentage
Elephantiasis, leg	39	26	65	51 2
Elephantiasis, forearm and hand	5	4	9	7 1
Elephantiasis, leg and arm	3	1	4	3 2
Filarial lymphangitis of leg or arm	24	25	49	38 6
TOTALS	71	56	127	.

Elephantiasis of the extremities forms the chief type of filarial disease met with in Patnagarh   History of lymphangitis of the extremities recurring periodical.y is elicited in every case of lymphatic obstruction   Abscesses along the main lymphatic regions or on elephantoid limbs are fairly common   Other manifestations of filarial infection such as elephantiasis of the genitals, hydrocele or chyluria

do not occur in Patnagarh   This is in agreement with the reports by Brug (1927), Korke (1929) and Iyengar (1932), in areas where ' *Filaria* ' *malayi* occurs

No distinct difference is seen in the filarial disease distribution between the sexes

A HOUSE TO HOUSE SURVEY

A thorough and detailed house to house survey was carried out in Ghasipara (ward No 11, *see* Map)   The huts are built of clay and mud and the roofs are thatched   They lie in three rows each hut being separate from the other   There are no roads, no drains nor any system of conservancy   The people belong to the sweeper class   The ' para ' forms the southern boundary of the town and is between two tanks—Ramsagar and Hirasagar   The results of investigation of this ' para ' are given below —

TABLE IV

Showing the microfilaria rate and filarial disease rate of Ghasipara

Age group	MICROFILARIA RATE				FILARIAL DISEASE RATE			
	MALE		FEMALE		MALE		FEMALE	
	Number examined	Number positive	Number examined	Number positive	Number examined	Number positive	Number examined	Number positive.
1–5	8	3	6	1	0	0	0	0
6–10	7	2	10	6	0	0	0	0
11–15	5	1	2	0	2	0	0	0
16–20	0	0	2	1	0	0	0	0
21–25	4	?	2	2	0	0	2	1
26–30	6	1	5	2	1	0	0	0
31–35	4	0	5	1	1	0	0	0
36–40	3	2	5	2	0	0	0	0
41–45	0	0	4	1	0	0	2	1
46–50	1	1	3	2	0	0	0	0
51–55	1	0	0	0	0	0	0	0
56–60	0	0	3	1	0	0	0	0
Over 60	0	0	2	1	0	0	0	0
TOTALS	39	12	49	20	4	0	4	2

The above results are in agreement with those obtained with random sampling, *vide* Tables I and II

TYPE OF FILARIAL INFECTION

As mentioned previously the infection observed in Patnagarh was entirely ' *Filaria* ' *malayi* Brug   The absence of *Wuchereria bancrofti* infection in this area is very striking   For the identification of the kind of filarial infection the following diagnostic table on the two species of human microfilariæ hitherto found in India may be of use

	Microfilaria bancrofti	*Microfilaria malayi*
Length	260μ to 300μ	145μ to 180μ
Breadth	7μ to 10μ	8μ
Sheath	Present	Present
Periodicity .	Nocturnal	Nocturnal
Disposition in a thick film	Smooth curves	Body with short wavy curves often presenting a crinkled appearance
Nuclei .	Small, evenly distributed, not overlapping	Larger, massed up and overlapping
Tail end	Tip of tail devoid of nuclei	Tip of tail irregular with 1, 2 or 3 minute nuclei

In India the distribution of ' *Filaria* ' *malayi* Brug has been found to be very discontinuous   It occurs extensively in the coastal areas of Travancore (Iyengar, *loc cit*) and patchy distribution of this infection has been recorded by Korke (*loc cit*) in Balasore, therefore the present record from Patnagarh is of interest, as these towns are within 200 miles of each other

MOSQUITO SURVEY

The following species of mosquitoes were found to be prevalent in Patnagarh during the period of the survey   The identification was kindly done by Captain P J Barraud, Officer-in-Charge, Central Malaria Bureau, Kasauli

Mansonia (*Mansonoides*) *annulifera*, *M   uniformis*, *Culex   fatigans*, *C* (*Culiciomyia*) *pallidothorax*, *Anopheles hyrcanus*, *A   pallidus*, *A   annularis* (*fuliginosus*) and *A   culicifacies*

The breeding of *Culex fatigans* and *C   pallidothorax* was found to be restricted to a solitary sump in a temple in Brahmanpara, and to a few rain-water collections in broken earthen pots lying scattered outside huts

Mansonoides spp and *Anopheles spp* were found to be breeding in the tanks of which there are sixteen in the town area   Most of the tanks lying on the outskirts of the town were found to be densely covered with Pistia

INTERMEDIATE HOST

A large number of mosquitoes collected from dwelling houses from different parts of the town were dissected and examined for filarial infection to determine the intermediate host   Over 200 specimens of different species were examined and the findings are furnished in Table V —

TABLE V

Mosquito dissections

Species	Number examined	Number found to
Mansonia (Mansonoides) annuliferus Th	110	23
M uniformis Th	23	1
Culex fatigans Wied	28	0
Anopheles hyrcanus Wied	6	0
A fuliginosus Giles	26	0
A pallidus Th .	20	0
A culicifacies Giles]	5	0

The most common species in the area was *Mansonia annul fera*   Natural infection was found in only two species *Mansonia annuliferus* and *M uniformis*   Judging from the numerical prevalence of *M annuliferus* and the high rate of infection observed under natural conditions it is evident that this species is the prime, if not the sole, agent concerned in the transmission of filarial infection in Patnagarh   *M uniformis* also plays an appreciable part in the transmission of the infection   The other species of mosquitoes are probably unimportant from the point of view of the transmission of ' *Filaria* ' *malayi* infection

SUMMARY

1   Patnagarh in Orissa, a small rural type of town, has endemic filariasis , the infection consists entirely of ' *Filaria* ' *malayi* Brug   The gross infection rate is 17 8 per cent and the filarial disease rate is 3 3 per cent   The types of filarial diseases are either filarial lymphangitis or elephantiasis of the limbs   Elephantiasis of the legs is the most common filarial affection   Elephantiasis of the genitals, hydrocele and chyluria are entirely absent

2 Transmission of the infection is primarily through the agency of *Mansonoides annulifera* which is the commonest species of mosquito in Patnagarh The species showed an infection rate of 20 9 per cent under natural conditions *M uniformis* plays a secondary part in transmission Species of *culex* and *anopheles* do not appear to be concerned with the transmission The breeding places of *mansonoides* are the large tanks (shown on the Map) densely covered with Pistia which occur in the area surrounding the town

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THE VIABILITY OF THE 'INFECTIVE' FORMS OF THE LARVÆ OF *WUCHERERIA BANCROFTI* WHEN FREED FROM THE MOSQUITO HOST [*]

BY

K P MENON, L R C P & S,

AND

P V SEETHARAMA IYER, M A

(*From the King Institute of Preventive Medicine, Guindy*)

MOSQUITOES infected with the larvæ of *Wuchereria bancrofti* become infective in about twelve days   On or about the tenth day, one often meets with two distinct kinds of larvæ, a shorter, sluggish form and a longer, active one   The shorter, sluggish forms are probably larvæ whose development has been arrested   They are seldom present in the proboscis   Their cuticle is not hardened and, when an infected mosquito is dissected, either in saline or distilled water, these stout, sluggish larvæ burst and die   A small herniation occurs in a few minutes at some weak spot in their body and this enlarges and bursts

The longer, active forms referred to above are what we consider the ' infective ' forms   These have a resistant cuticle and appear in the proboscis generally in pairs   These active larvæ retain their morphological characters intact even after death   As we found that these forms are able to live in saline and other fluids for a considerable time, it was decided to test their viability outside the mosquito in various fluids of known strength   The possible importance which might attach to this information lies in the fact that it suggests methods of filarial infection of man through channels other than the bite of infected mosquitoes   So far as we know, there is no record of any experimental observation on this subject in the published literature

During the course of a filariasis survey of Saidapet, materials and opportunity were available to us for the study of this problem

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[*] Paper read at the 23rd Session of the Indian Science Congress held at Indore in January 1936

Naturally infected mosquitoes (*Culex fatigans*) were caught in houses in Saidapet, an area where filariasis is endemic   They were killed by concussion in test-tubes and dissected on glass slides in normal saline   Infective forms of larvæ, when seen under the microscope, were pipetted off and placed in well-slides containing the various fluids in which the survival period of these larvæ was ascertained   Observations were made in relation to plain tap water, water containing 0 2 per cent hydrochloric acid, 0 5 per cent and 0 25 per cent lactic acid and water containing starch and egg albumen, with and without hydrochloric acid

The following table indicates the various fluids, the number of observations in each fluid and the duration of life of the infective larvæ in each of the fluids ·—

TABLE

	Reagents	Number of observations	Period of survival	
			Maximum	Minimum
1	Tap water	7	6½ hours	4½ hours
2	Ringer's solution	1		3½ „
3	0 2 per cent hydrochloric acid	3	12 minutes	10 minutes
4	0 5 per cent lactic acid	1	Immediate death	
5	0 25 per cent lactic acid	1	„	
6	0 2 per cent HCl + 2 5 per cent to 10 por cent egg albumen	6	45 minutes	20 minutes
7	0 5 per cent lactic acid + 2 5 per cent egg albumen	5	55 minutes	25 minutes
8	15 per cent egg albumen in tap water	1		4 hours 15 minutes
9	4 per cent egg albumen in tap water	1		6 hours 30 minutes
10	2 per cent starch solution + 0 2 per cent HCl	5	15 minutes	10 minutes
11	2 per cent starch solution	3	5½ hours	2 hours

It was found that the ' infective ' larvæ could live in tap water from 4½ to 6½ hours   This shows the possibility of the ' infective ' filarial larvæ remaining alive in natural waters, on liberation from their insect hosts into water either at the death of the latter or by emergence from their proboscides at the time of egg laying The period of survival in water is also sufficiently long to warrant the inference that human hosts could contract the infection through drinking the water or

through the skin, were such a method possible   It was also found that the larvæ could live in 0 2 per cent hydrochloric acid with different concentrations of starch and egg albumen for 20 to 45 minutes   This would appear to suggest the possibility that the larvæ when swallowed could resist the action of the acid gastric contents

These observations are reported in the hope that they may lead to further investigation along similar lines with a view to settling whether filarial infection with *W bancrofti* may be contracted by means other than the bite of the mosquito

[The following quotations from Manson are of interest in this connection —
1   ' That the young filaria can live in water for a time is certain   it is conceivable that some of them, such as those which at the completion of their stage of development in the mosquito find their way into the abdomen of the insect, may escape into water when the mosquito lays her eggs or dies '   (Manson's ' Tropical Medicine ', 6th ed , 1912, pp 609-610)
2   ' In my original observations on this subject in 1879 and 1883 (*Trans Linn Soc*, 1883)   I conjectured that the fully metamorphosed filaria escaped from the insect either at her death or when she deposited her eggs and that thus, in drinking water, it obtained a chance of gaining access to the stomach of the human host   ' (Manson's ' Tropical Medicine ', 6th ed , 1912, p 610—footnote) —*Editor, I J M R*]

LIFE-HISTORY AND MORPHOLOGY OF *BABESIA CANIS* IN THE DOG-TICK *RHIPICEPHALUS SANGUINEUS.*

Parts I– II.

BY

LIEUT -COLONEL H E SHORTT, I M S,

Director, King Institute of Preventive Medicine, Guindy, Madras

Part I.

THE LIFE-CYCLE OF *B. CANIS* IN *R. SANGUINEUS.*

I INTRODUCTION

THE genus *Babesia* is notable on account of the fact that the transmission from host to host of infections with one of its members, *B bigemina*, by ticks was the first instance to be described of the transmission of a protozoal parasite by an arthropod This discovery by Smith and Kilborne (1893) opened up the vast field of research on the part played by arthropods in the transmission of protozoal diseases which has since that date revolutionized our knowledge of tropical diseases

While the most important aspect of this discovery was the light it shed on a principle of nature its more direct effect, leading to the study of the families *Babesiidæ* and *Theileriidæ*, has been to reveal the enormous economic importance of these families throughout the world and over a wide range of animal life both in nature and under domestication

Among domestic animals which suffer from the various forms of Babesiasis or Theileriasis caused by different species of the families mentioned are to be cited cattle, horses, sheep and dogs and, as the disease in some of its various forms is present in every continent of the world and at times produces serious losses in domesticated animals, its economic importance need not be further stressed

The species which forms the subject of this communication *Babesia canis* was first recorded by Piana and Galli-Valerio in Italy in 1895 as occurring in dogs and has since been recorded from various parts of Europe, Africa and Asia It is at least doubtful whether it occurs naturally in America and Australia

In India it was first recorded by Christophers (1904) and later by James (1905) and since that date has been recognized as a very important disease of dogs and the cause of much mortality, especially among imported breeds

Lounsbury (1901, 1904), in South Africa, was the first to prove that the tick was the arthropod host, and he also demonstrated, following Smith and Kilborne's original work, that the infection passed through the eggs of the tick to the succeeding generation of adults

The carrier in India is the dog-tick *Rhipicephalus sanguineus*, but other species of tick have been proved to be the vectors in other parts of the world

Although the transmission of *B canis* infection by the dog-tick was demonstrated, as has been stated, in 1901 and the subject has been investigated by many distinguished workers, notably Christophers, Marzinowski and Bielitzer, and later by Regendanz and Reichenow it is only now, after the lapse of over thirty years, that the full cycle of development of this parasite in its arthropod host is becoming known

Probably one of the chief reasons for this slow accumulation of facts is the technical difficulties inherent in the histological examination of ticks Christophers' original work appears to have been carried out entirely by examination of fresh preparations and of fixed and stained smears and it is a tribute to the remarkable accuracy of his deductions from these preparations that, in the present work, which has been carried out almost entirely by means of microscopical sections, his conclusions with regard to the various appearances found by him have been proved to be almost invariably correct The later workers mentioned have made full use of the method of microscopical sections in confirming their observations on fresh material and fixed smears but, so far as can be gathered from their own descriptions, have been obliged, at least in certain stages of the ticks investigated, to remove from the bodies of the ticks the organs being examined before submitting the latter to section cutting

This has been rendered necessary chiefly on account of the difficulty of successfully cutting thin sections, both of the soft parts and of the tough chitinous covering of the tick This difficulty is not entirely evaded by the previous removal of the dorsal chitinous shield of the tick, which can be successfully accomplished without undue disturbance of the underlying viscera

In the present work every effort has been made to base all observations on the exact localization of the parasites in the body of the tick only on preparations in which the body contents of the tick have been allowed to remain *in situ*, the only concession made to the difficulties in technique being, in the case of the adult and nymphal stages, the removal of the dorsal chitin without disturbing the relative positions of the various internal organs The larval stages have invariably been cut whole

II PREVIOUS WORK ON THE LIFE-HISTORY OF *B CANIS* IN TICKS

Until the recent appearance of an excellent piece of work by Regendanz and Reichenow (1933) there has been very little published work on the life-cycle of *B canis* in dog-ticks since the memoir by Christophers (1907) The possible reason for this neglect has already been stated

Christophers' study of the subject elucidated the main points, both in regard to the stages of the tick which induced infection in dogs, including the occurrence of hereditary infective power in the ticks, and in regard to the actual stages in the life-cycle of the parasite itself

As regards the former point the writer has been able to confirm Christophers' conclusions with regard to the infectivity of the adult and nymphal stages and the occurrence of hereditary infections through the ova to the next generations of ticks In addition, it has now been proved that the larval stage is also capable of producing infection and it has been possible to confirm the deduction of Christophers, based on his experiments, but of which he had no definite proof, that stage to stage infection occurs By stage to stage infection is meant that the tick, infected as a larva, transmits the infection as a nymph and one infected as a nymph transmits the infection as an adult

As regards Christophers' account of the various stages in the life-history of the parasite itself the description to be given here will show that, in the main and, as already stated, to a remarkable degree, considering that Christophers worked without microscopical section, the present study confirms his findings

Certain details differ from his interpretation of some of the stages in the life-history but the latter is so complicated that it is possible further research may reconcile these differences The only major point on which the writer does not agree with his description is in his localization of the developing forms of the parasite during the interval between their passage through the gut wall and their appearance in the salivary glands He localizes them at this stage in the undifferentiated embryonic tissues of the nymph or adult, tissues which may eventually form salivary glands, muscle, etc This would appear to leave to pure chance the final localization of the parasite, whereas, to produce infection, it is essential that the final localization should be in the salivary glands It is in this way that Christophers explains the occurrence of the parasites, not only in the salivary glands but in muscle tissue and elsewhere Even here, however, it is only fair to say that Christophers stated that it was unlikely that all the parasites found in areas other than the salivary glands were doomed to degenerate or lie idle and that probably some provision existed for their transference or migration to the glands Without going into details of the writer's views here, as they are fully elaborated later, it is only necessary to add that an entirely different conclusion was come to from a study of microscopical sections of ticks made at different stages of infection

Regendanz and Reichenow (*loc cit*) in their excellent account of the life-history of *B canis* in *Dermacentor reticulatus* have given a description which corresponds fairly closely with the present findings but which differs in certain details Thus, they describe the earlier stages of development of the parasite as taking place in the cells of the tick's gut This has not been the writer's experience as the account below will show Thus the earlier stages of development were found to take place in certain phagocytic cells in the body cavity It is possible that both interpretations are correct since the present study deals with a different species of tick and chiefly with the nymphal stage of it, whereas they appear to have done most of their work with the adult stages One other aspect in the life-history which formed a characteristic feature at a certain stage in the case of the parasite investigated here, viz, the concentration of parasites in the muscles and muscle-sheaths of the

tick at a certain stage, was either not seen or has been completely ignored by them Here again the discrepancy is possibly due to the different species of tick

Other points of difference between the present work and that of Christophers, and of Regendanz and Reichenow are in minor points of detail and will emerge in the course of the description so that they need not be considered at this stage

Besides these two important studies on *B canis* there has been some important recent work on other species of *Babesia*, notably that of Dennis (1932) on *B bigemina* and of Cowdry and Ham (1932) on *Theileria parva* As both these studies deal with a different species of *Babesia* and the life-history is associated with ticks (*Margaropus annulatus* and *Rhipicephalus appendiculatus*), the habits of which differ markedly from those of *R sanguineus*, it is not remarkable that there should be many more points of divergence between the writer s findings and theirs than in the case where the former findings were compared with those of other workers studying the same species of *Babesia*

Any points in the life-history of *B bigemina*, as elucidated by these workers, and which are relevant to any of the present findings will be referred to in the description and need no further mention here

III OBJECTS OF THE PRESENT INVESTIGATION

At the time this work was commenced the paper by Regendanz and Reichenow referred to above had not appeared and the most complete account of the life-history of *B canis* in the dog-tick was still that of Christophers The object aimed at, therefore, was to try and fill in the lacunæ left by Christophers, small though these have been shown to be, and to present as complete an account as possible of the life-history of *B canis* in *R sanguineus* To this end the method of microscopical sections was used in order accurately to delimit the localization of the parasite at its different stages in the tissues of the tick in a manner, and with an exactness, only possible by this method The work of Regendanz and Reichenow might be thought to have fulfilled these objects, and in many respects, the present findings confirm theirs but in certain respects, some of which have been referred to above, the writer's observations differ from theirs sufficiently to make the recording of them necessary It is with the object, therefore, of confirming the work of Regendanz and Reichenow, where their observations coincide with the writer's and of stating his own findings where these differ from those of the authors mentioned that this account is now presented It may eventually prove that the discrepancies in the two descriptions of the life-cycle of *B canis* are due to differences caused by habitat in a different tick host although one is inclined to believe that this is unlikely and that they are due to differences in interpretation of the appearances seen and that one or other is correct and not both

IV MATERIALS AND METHODS

Ticks —All the work has been carried out with the common dog-tick of India *Rhipicephalus sanguineus* This is easily obtainable from street dogs but care has to be taken that the ticks when collected are not injured This tick normally has three hosts Each stage, larva, nymph and adult, feeds to repletion on a dog and then drops off It crawls, most frequently upwards, to some sheltered crevice and

there becomes quiescent to digest its blood meal   This is followed, in the case of the adult female, by oviposition and in the case of the larva and nymph by the subchitinous changes leading to the next ecdysis, nymph or adult as the case may be It is seen from this life-history, which has been too often described to be gone into in greater detail that, if the ticks are removed from the dog, they are likely to be incompletely fed and, in any case, likely to be injured so that it is best to collect the engorged adults from their hiding places and to breed a stock of ticks from their eggs   Another method is to enclose the dog in a cage which is itself enveloped in a large calico bag   The engorged ticks of all stages may then be collected from the bag as they drop off

The engorged adults, if kept in test-tubes with pieces of crumpled tissue-paper, lay large clusters of eggs which hatch into thousands of larvæ   These can be fed on dogs, collected from the calico bag, and placed in test-tubes till they become nymphs which are similarly treated and we again reach the adult stage   In this way a continuous stock of clean ticks in all stages can be maintained

Christophers' work was in some cases complicated by the fact that he had some cages which were infested with ticks   These ticks were in some cases normal and in others infected, so that an experimental clean dog placed in the cage might become infected by the bites of the parasitized ticks and thus infect the clean ticks The result of this was that the individual ticks removed from such a cage might show the *Babesia* infection in different stages and it was even possible that two different stages of infection might be present in the same tick   In order to obviate any such difficulties and to render more easy the exact correlation of different stages in the development of the parasite with known periods after the feed of the tick on an infected dog the writer has been careful always to put only one stage of tick at a time, i e adult, nymph or larva, on an infected dog   In this way the stages of *B canis* found in any tick could be correlated very exactly with the time which had elapsed since the tick became infected by feeding on the dog   This correlation could not be absolutely exact unless a single tick only was fed because, whereas some ticks attach themselves at once on being given the opportunity, the majority do not   The result of this is that out of a large batch of nymphs placed at the same time on a dog it may be several days before all which are going to feed are attached and, therefore, in a single batch of infected nymphs there may be a difference of a similar number of days in the stages of development of *B canis* in individual ticks

Dogs —It is a widely-held belief that imported European breeds of dog are much more susceptible to infection with *B canis* than the indigenous dog of India One is not quite sure that this belief is justified as regards mere susceptibility to infection but it is probably the case that European breeds of dog take the disease more severely and are more liable to die as the result of it if treatment is withheld In the case of indigenous dogs it is probable that a large number are infected as puppies and, as experimental infections show, it is almost certain that a large proportion of these will die of the infection   Those which survive the initial acute attack seem to acquire a degree of immunity or at least tolerance and in these a chronic infection is set up which may persist for a very long time   This condition is more likely to be found in adult dogs and the presumption is that they became infected as puppies and have retained a low grade infection   Such dogs are usually seen to be somewhat anæmic but, on some occasions, only the chance finding of

parasites in blood films has revealed the presence of an unsuspected infection   The whole of the work here described has been carried out with indigenous dogs and this is probably responsible for the impression gathered from reading Christophers' reports that, on the whole, he was accustomed to deal with heavier infections both in his dogs and his ticks than was the case in the present series, a result probably due to the fact that he largely utilized imported breeds of dogs

Microscopical and section-cutting technique

Section-cutting technique —Throughout the work the embedding method employed has been the method of double embedding in celloidin and paraffin   The full details of the technique need not be gone into but the method employed may be broadly outlined as a guide to others in the somewhat difficult problem presented by ticks

Fixation —Many fixatives were tried but those which have given the most uniformly successful results, and which have been employed almost exclusively in this investigation, are the fixatives of Bles, Carnov (formula with chloroform) and Bouin

In order to get satisfactory fixation in nymphs and adults it is necessary, as a preliminary, to remove the dorsal chitin   This may be done by fixing the ventral surface of the tick on a block of paraffin, the tick being stuck on an area melted with a hot spatula   A circular retaining wall of plasticine is made round the tick and the chamber so formed is flooded with saline solution or with the fixative to be used   The chitin is cut with scissors or with an iridectomy knife or safety-razor blade around the lateral margin of the tick and is then lifted off with suitable needles in handles, retaining fibrils of tissue being broken through with the needles or knife   Alternatively, the tick may be held in the fingers before fixing on the paraffin block and the lateral margin cut round as described   The dorsum being removed the tick is transferred to the fixative selected

Fixation being complete it is dehydrated in alcohols, transferred to clove oil till clear, and then to a solution of celloidin in clove oil   In this it remains a minimum of four days but preferably for a week or more   It is then transferred, in a drop of the celloidin solution, to a paraffin-coated cover-slip which is floated upside down on chloroform until the button of celloidin containing the tick detaches itself   The celloidin-embedded tick is transferred to fresh chloroform to clear and harden and then, without drying, is embedded in paraffin   For cutting purposes the ticks are oriented so as to cut tangential sections of the dorsum, thus avoiding, as far as possible, the hard chitinous parts on the ventral aspect

The technique outlined above applies to the adult and nymphal stages only of the tick   The larvæ are very minute forms and not easily dealt with by the same methods   As very large numbers have to be examined by the method of microscopical sections one had to devise a different process for larvæ, one by which many individuals could be cut and examined together   This was rendered the easier by the fact that satisfactory fixation could be obtained of whole larvæ without the necessity of removing the dorsal chitin   The procedure adopted with complete success was as follows —

In a small rectangular piece of amyloid liver a small pocket is excavated with an iridectomy knife or a sliver of a safety-razor blade   Into this are inserted about

one hundred partially fixed tick larvæ   The liver containing the larvæ is immersed
in the fixative and the mouth of the pocket is closed with a drop of egg albumen
which is poured in through the fixative   The albumen also overlaps the edges of
the pocket and is immediately coagulated by the fixative resulting in the effective
sealing of the mass of larvæ in the pocket of liver   Fixation of the larvæ is quite
satisfactory and the block of liver is cut either vertically to the plane of the mouth
of the pocket or tangentially, orientation being facilitated by the rectangular shape
of the liver   In the former case the closely packed larvæ are seen surrounded by
liver on three sides and in the latter completely surrounded   By using this method
it is obvious that the larvæ will present every variety of orientation but, as individual
larvæ can, by their position relative to their fellows and to the liver walls, be followed
from section to section every part of every larva can be examined in a preparation
cut as serial sections   Text-figure 1 shows the arrangement described —

PLUG OF
ALBUMEN

— CELL OF
LIVER
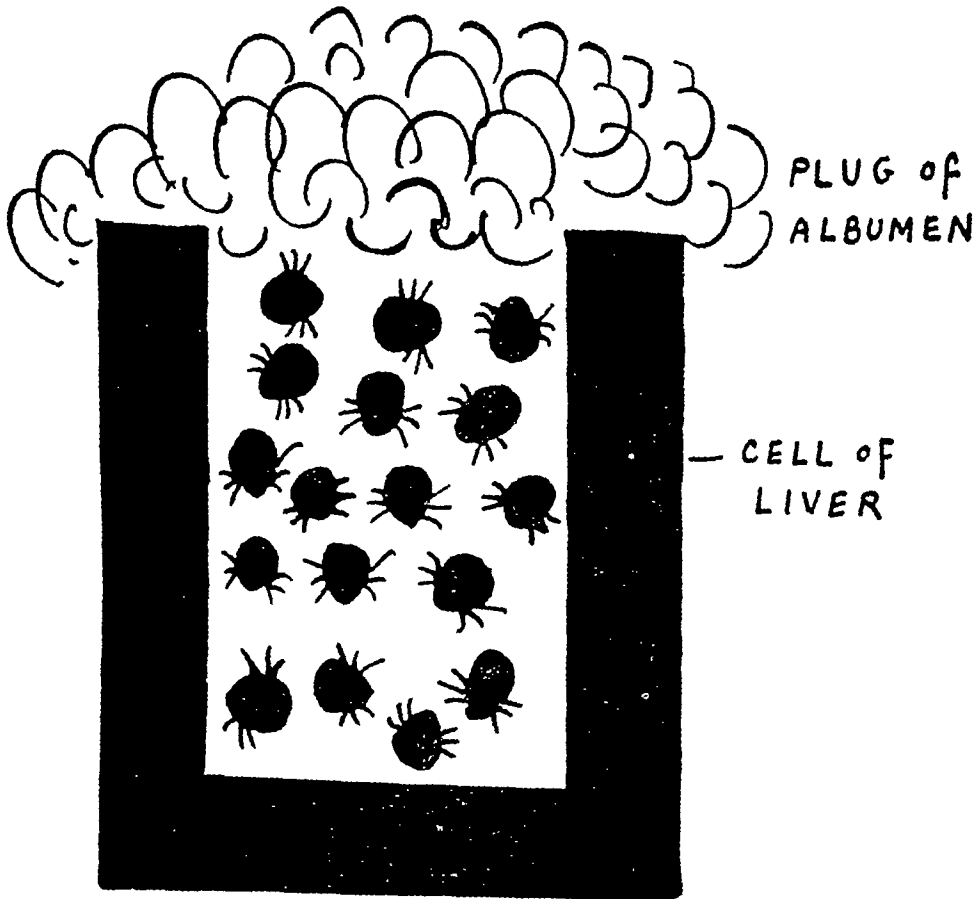
TEXT FIGURE 1;

Staining technique—In all protozoological work in connection with arthropods
the writer's experience has been that two stains, Giemsa or some of the other
modifications of Romanowsky's stain, and iron hæmatoxylin are so greatly superior

to all others that only for exceptional and restricted purposes need one go beyond these two stains

In the present investigation Giemsa's stain has always been used when one has been searching for stages in the life-history of *B canis* which were not known and familiar   The polychrome results obtained by this stain greatly facilitate search for unfamiliar forms especially when normal appearances are well known to one   Once a particular stage in the life-history was recognized with certainty and its morphology roughly elucidated as brought out by Giemsa's stain specimens of the same stages, stained with iron hæmatoxylin, were made as permanent preparations   For this purpose a frequent procedure was to stain one half of a tick cut in serial sections with Giemsa's stain and the other half with iron hæmatoxylin   Similar stages in the same individual tick were thus capable of being compared as shown by the two stains   With the iron hæmatoxylin a counter stain, dissolved in carbol-xylol, was frequently used   This counter stain was used immediately after dehydration and before clearing the sections in pure xylol   One method was found particularly useful in cases where a section stained with Giemsa showed some particular form which it was doubtful if one could obtain another specimen of   As this process does not seem to have been described elsewhere, and as it enables a permanent preparation to be made in place of a Giemsa-stained one which would eventually fade, even if kept in the dark, it may be useful to mention it here

A Giemsa-stained slide showing a form of which it is important to have a permanently stained record may be readily stained with iron hæmatoxylin   The slide is suspended in xylol in such a way that the cover-slip can slip off as the xylol dissolves the balsam or other mountant   The time taken will vary with the extent to which the latter has dried   The slide is next transferred, upside down, to a dish of methyl alcohol and allowed to remain until completely or almost completely decolorized, precautions being taken to prevent undue evaporation of the alcohol

The preparation is now transferred to a solution of iron ammonium alum and the subsequent operations are those ordinarily employed in the iron hæmatoxylin technique

V   DESCRIPTION OF THE DIFFERENT STAGES OF *B CANIS* AT DIFFERENT PERIODS IN ITS LIFE-HISTORY

The stages gone through in the life-cycle vary to some extent according to whether the parasite is ingested by the adult or the immature forms of the tick as, it will readily be understood, is necessarily the case where the infection may be hereditarily transmitted through the eggs but is not always so transmitted

(A) *If ingested from infected dog's blood by the nymphal stage of the dog-tick* R sanguineus —The stage of the tick most readily investigated is the nymph   This stage being intermediate in size between the relatively enormous fully fed adult and the minute larva is in every way the most suitable for the manipulations and examinations, both macroscopical and microscopical, to which the ticks have to be submitted   It has been the form, therefore, most extensively used in this study from the point of view of the contained parasites and in the description given below will be considered first, while the larval, adult and egg stages will be subsequently considered only in so far as they affect differentially the stages in the life-cycle of *B canis* contained in them

For the purposes of a description of the life-cycle then, one may commence with the changes in *B. canis* initiated at its ingestion by the nymphal stage of the dog-tick and follow the evolution of the parasite up to the point where the nymph becomes the adult tick. At this stage a point is reached where no further development occurs in the parasite until the adult tick commences to feed and therefore, for convenience of description and to carry the sequence of stages in the life-cycle of the parasite to their conclusion in the infective stages in the salivary glands, one may continue by describing the changes which occur after the adult commences feeding. This may be said to conclude the description of the full life-cycle in the case where the nymphal stage of the tick becomes infected by feeding on an infected dog. Subsequently, the changes occurring when the adult and larval stages are the ones originally acquiring the infection will be similarly, although more shortly treated, special stress being laid on those stages necessarily differing from the stages described as occurring when the nymph is primarily infected.

By this procedure it is hoped to make as clear as possible, so far as it has been elucidated, the complicated life-cycle undergone by *B. canis* in the tick. In actual practice, however, one has fallen short of this ideal inasmuch as it has not been found possible to observe in sections the earliest stages of development, i.e., those stages immediately succeeding the ingestion of the parasites by the tick in its blood meal and one has to be content with commencing the life-history of *B. canis* in the tick at a stage where some days have elapsed after the nymph has completed its blood meal and dropped off the infected dog.

(a) *Appearances shown by* B. canis *four or five days after the nymph of* R. sanguineus *has completed its meal and dropped off the infected dog.*—If a section of a nymph at this stage is examined under a low power of the microscope it will be seen that under the chitinous cuticle there is a single layer of cells which tend to be columnar or cubical. Under these there is the diffusely-located fat body, while, still more internally, there is the gut, containing the partially digested blood meal. In the anterior part of the tick the layer of cells referred to tends to become several cells thick, possibly an optical effect due to tangential sectioning where the limb buds are forming. This layer of cells, which may be referred to as the epidermal layer, is composed of cells which will produce on its outer surface the non-cellular chitin of the adult (Plate XXXI, figs. 1 and 2).

In an infected tick, if the epidermal layer of cells is followed around the body there will be found at intervals, varying with the degree of infection, lying underneath it and usually closely applied to it, certain large cells which contain in their interiors an early stage of development of *B. canis*. These cells and their nature will be discussed at a later stage.

The actual stage of development reached by the parasite at the period we are considering will vary considerably, not only in different ticks but in different cells in the same tick. The earlier form is that of a globular body with a single small chromatic mass more or less centrally situated. The body is smaller than the nucleus of the containing cell. The cytoplasm of the parasitized cell may show some rarefaction in the form of a halo round the parasite. At a slightly later stage the nucleus of the parasite will be found to be much more bulky and commencing division is indicated by its lengthening shape. It is not infrequent for a single cell

to contain two such forms, not necessarily at the same stage of development (Plate XXXII, fig 3)

The next stage of development is marked by the continued division of the chromatin of the parasite until the latter is filled with irregular masses, varying in size and many of them apparently connected together by fine threads of chromatin This process is accompanied by a great increase in the bulk of the parasite, which may now fill most of the cell and begin to displace the nucleus, which it now greatly exceeds in size, to the periphery of the cell   It is not infrequent for two such large parasites to be present in one cell, a seemingly natural consequence of similar double infections at the stage previously described (Plate XXXII, figs 4, 5 and 6)   In certain cases the nucleus of the containing cell may not be visible so that the simulation of a true cyst is produced

At this stage of development of the parasite the containing cell is very frequently found to contain comparatively large and somewhat faintly staining globules or granules   These stain homogeneously, and vary in size in different cells but their nature is not known (Plate XXXII, figs 4, 5 and 6)   The cell and its contained parasite or parasites may, for convenience of description, be called the pseudocyst

The stage just described is followed by one in which the cytoplasm of the parasite within the pseudocyst commences to be aggregated into clumps   The cytoplasm stains darkly and this results in a considerable masking of the granules of chromatin but the general arrangement, although obscured, seems to be for the particles of chromatin to be the centres round which the cytoplasm aggregates (Plate XXXII, fig 7)   This stage is evidently the beginning of the series of changes to be next described resulting in the formation of one of the most characteristic and easily observed stages in the evolution of the parasite owing to its large size, viz , the fully developed pseudocyst

(*b*) *Appearances shown by* B canis *about seven days after the nymph of* R sanguineus *has completed its meal and dropped off the infected dog* —The stage of development reached by *B canis* seven days after the dropping of the nymph from its dog host is extremely characteristic and, on account of its large size, is readily found in infected specimens   This is further facilitated by the fact that the pseudocysts lie generally closely under the peripheral epidermal layer of cells previously referred to, in some cases closely applied to it, and in many cases actually indenting it so that the cells in immediate apposition to the pseudocyst are shortened, and made cubical instead of preserving their typical columnar form (Plate XXXII, figs 8 and 9, and Plate XXXIX, fig 44)

It is not infrequent for the pseudocysts, in heavily infected specimens, to lie in groups under the epidermal layer, closely applied to one another   In such cases the pseudocysts not only indent the epidermal layer of cells but may themselves be deformed and facet each other (Plate XXXIII, fig 10)   In pseudocysts at this stage of development the nucleus of the containing cell is always peripheral, being pushed into this situation by the continued growth of the parasites, and may or may not be visible in any individual case   The central position apparently occupied by the nucleus in certain pseudocysts is owing to their being seen in optical section An instance of this will be noticed in Plate XXXIII, fig 10

In the description given in the last section the pseudocyst had reached the stage where the cytoplasm was beginning to aggregate into masses

In the stage reached seven days after the nymph has completed its meal this process has been completed and a typical mature pseudocyst has the following appearance —

The shape tends to be spherical or ovoid but, owing to the compressibility of the contents, the regular contour may be deformed to conform to the space within which the pseudocyst is lying   This is well exemplified in Plate XXXIII, fig 10, where several are shown varying in shape due to compression by their surroundings or pressure against each other

Each is surrounded by a distinct wall representing the cell wall of the parasitized cell and in no way connected with the contained parasites   At one side of this cell wall the nucleus of the parasitized cell may or may not be visible but is always obviously compressed by the contents

These contents take the form of a large number of spherical or ovoid bodies each containing a single nucleus   These bodies vary to some slight extent in size but there is a distinct tendency for those within any individual pseudocyst to be uniform in size

The smaller sizes are definitely uninucleated and probably do not increase further in numbers   The large sizes occasionally may show some lengthening of the nucleus possibly indicating division to form the smaller sizes but we cannot say that we have actually seen dividing stages and are rather inclined to believe that the number of bodies is primarily dependent on the subdivision of the chromatin described in an earlier section at the stage previous to the condensation of cytoplasm around the chromatin granules

The number of individual parasites contained in a single pseudocyst appears to vary considerably but must in some cases certainly be two or three hundred   In certain cases they seem to fill the pseudocyst, being closely applied to one another and to the containing wall, while in other cases they are more scantily disposed and considerable free space is left both between the parasites themselves and between their main bulk and the containing wall   Occupying the spaces left vacant between the parasites and between them and the containing wall there is usually a coarsely granular debris which is presumably the degenerated cytoplasm of the original host cell coagulated by the fixative

In some instances a pseudocyst such as has been described contained, in addition to the multiple bodies, a single very large parasite at a much earlier stage of development   Such accompanying parasites have been seen at the stage before fragmentation of the chromatin (Plate XXXIII, fig 11) and at a later stage where fragmentation is well advanced but condensation of the cytoplasm around the fragments has not occurred

At first, appearances such as these inclined us to consider the possibility that two such dissimilar forms in a single pseudocyst represented sexual differences, especially as it was comparatively frequent to find two parasites in one cell in the earlier stages of development   Further study, however, gave us no grounds for regarding such appearances as other than the accidental occurrence in one cell of two parasites at different stages of development

The stage to be next described represents the further development of the pseudocysts described above

It is possible that this stage should be described in the next Section as it may usually be a later development than that met with seven days after completion of its feed by the nymph but, as it has definitely been seen at this period, it is included in this Section

The contents of the pseudocyst as described above are found in the later stage to have undergone considerable developmental changes   The individual parasites have increased in size and the pseudocyst as a whole may, although not necessarily so, be larger   Besides increase in size the great majority of the parasites now appear to be crossed by one or occasionally two achromatic lines   In some cases these achromatic lines do not stretch right across the parasite while in other cases they appear actually to dissect off a portion of it   This, however, is only an optical effect due to the orientation of the parasite and its considerable depth of focus (Plate XXXIII, fig 12)
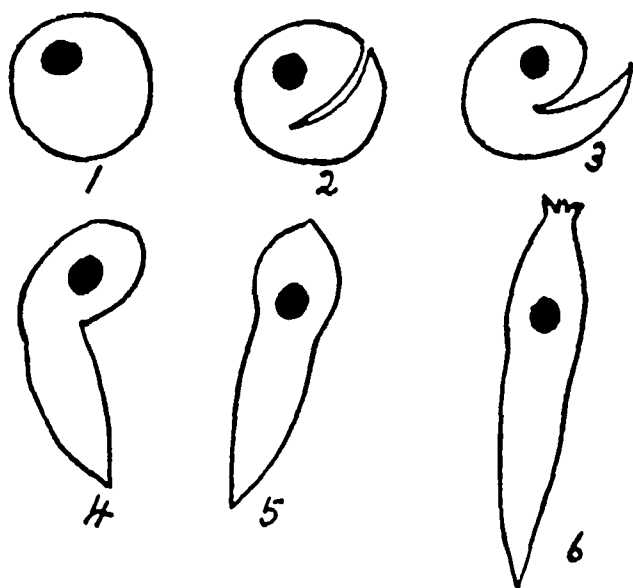
TEXT-FIGURE 2

A careful study of a large number of the individual parasites will reveal the fact that the achromatic line stops short of dividing the parasite into two but converts it into a larger bulky portion invariably containing the nucleus, attached to a smaller more attenuated portion which is closely applied to the neighbouring part of the larger portion   The appearance simulates the effect which would be produced were a tadpole to bend its tail back upon its head   The nucleus is invariably single   The stage here described is obviously that described by Christophers (1907) as leading to the formation of the club-shaped bodies, although he described these as being formed inside the gut   According to his description the achromatic line actually represents a cleft in the parasite which is later more widely opened by a swinging out of the smaller section the movement being completed by the latter coming eventually to lie in the same line as the larger portion to form a club-shaped body   The sequence of events may be represented diagrammatically in Text-figure 2

The correctness of his interpretation will be evident from the description of the next stage of development in the Section to follow

(c) *Appearances shown by* B canis *eleven to fifteen days after the nymph of* R sanguineus *has completed its meal and dropped off the infected dog*—The first stage to be described in this Section is that immediately following the form described at the end of the last Section

This consists of pseudocysts containing club-shaped bodies in various stages of maturity (Plate XXXIV, fig 13)   The development from the body with the achromatic line to the fully formed club-shaped body must be comparatively rapid as definite intermediate forms have not been found   The only differences seen in the clubs are degrees of longitude and breadth   The more mature club-shaped forms tend to be longer and with a more uniform outline than the younger   The latter are inclined to be more 'bulgy' at one end and attenuated at the other, a form associated with the mode of origin of the bodies   This development is evidently a provision for active migration of the parasites in the tissues of the tick host since the club-shaped bodies in fresh preparations are motile   Whether they leave the pseudocyst by boring their way out or whether they are liberated by its rupture we have not been able to determine but club-shaped bodies may, very rarely, be seen free in the tissues and it is evident that such forms are migrants on their way to a new location   This location is in nearly every case the muscles or muscle-sheaths This invasion of muscles has actually commenced and established itself at the stage we are considering but, as it reaches its fullest development in the adult tick, we shall describe it in the next Section which deals with the tick after its metamorphosis into the adult   At this stage it may be convenient to consider the question as to the nature of the tick cells which, from the earlier stages of development of *B canis* up to the liberation of the club-shaped forms, contain the various stages of the parasite   The question immediately arises as to whether these cells in the first instance ingested the parasites by phagocytic action as soon as the latter had penetrated the gut wall of the tick and gained the body cavity or whether the parasites by their own activity were able to penetrate the cells and so gain a safe intracellular habitat   The latter hypothesis would seem to imply a selective action on the part of the parasites resulting in the parasitization of the right type of cell only   The situation of the cells in question in close relationship to the fat body would suggest the possibility of their being œnocytes but the writer is not aware that the latter have ever been regarded as phagocytic cells and as such, from their action in engulfing the parasites, whether actively or passively, the parasitized cells in the tick must be considered to be   These cells, while typically situated close under what has been termed the epidermal layer may sometimes be found in other situations in the body cavity and are analogous, therefore, in character to the wandering phagocytic cells of vertebrates and from these considerations it appears likely that the cells must be considered hæmatocytes   This question will be touched on later when considering the parasitization of the muscles of the tick

(d) *Appearances shown by* B canis *twenty to twenty-one days after the nymph of* R sanguineus *has completed its meal and dropped off the infected dog*—At the time indicated the nymphal tick may or may not (depending on the temperature) have moulted and emerged as the adult tick   Even if the metamorphosis is not complete the tissues and organs of the adult will have so far developed within the old nymphal skin that the adult, just short of emergence, and the newly emerged adult may

be considered as essentially identical   For the purposes of our description we may consider the stages of *B canis* seen in a newly emerged adult tick   A very short examination will reveal that a complete change in the localization of the parasites has taken place   The parasites which, from the early stages of their development have been contained in pseudocysts, chiefly under the epidermal layer, are no longer so

Practically all the parasites are now found to be contained either in the muscle substance or the muscle-sheaths

So precise is the localization of the parasites at the time of emergence of the adults, and so uniform a finding was it in this investigation, that one cannot but consider that muscle-tissue, including the muscle-sheaths, is the site of election for this stage of the parasite

As we have already noted this invasion of muscle commences from about the eleventh day after the dropping of the nymph from the infected dog and it reaches its maximum development at about the time of emergence of the adult tick from the old nymphal skin   The appearances to be described below give a general account of the forms in which the parasite is found in the muscles and muscle-sheaths at any stage between these periods   While the strict selective affinity for muscle displayed by the parasites at this stage has been emphasized it should be mentioned that cyst-like aggregations are occasionally seen in various situations in the body cavity (Plate XXXIV, fig 11)   These are either contained definitely in cells and may even represent pseudocysts of the stages previously described or they may be bulging portions of muscle-sheaths containing parasites which have been cut tangentially to the muscle and so appear to have no connection with it   Serial sections have shown that in some cases at least this is the actual explanation of the appearances seen

(c) *Appearances seen when the parasite is in the muscle-sheaths* —The muscles of the tick are surrounded by delicate sheaths consisting of a single layer of cells   The arrangement is well seen in a cross section of a muscle (Plate XXXIV, fig 15) It will be noted that the cells forming the sheath bulge out opposite their nuclei and thin out peripherally   This thinning out may be so pronounced that two contiguous cells are connected by a delicate membrane which cannot be much more than the apposed outer and inner cell surfaces

Where two muscles are in close apposition it is presumed that each has its independent sheath on the apposed surfaces and this is apparently confirmed by the distribution of the sheath cell nuclei in such situations (Plate XXXIV, fig 16)

The parasites in the muscle-sheaths appear to be all intracellular and they may be present in sufficient numbers greatly to distort the normal outline of the sheath cells   This distortion may take the form of a pronounced bulge outward of the sheath to enclose the parasites (Plate XXXIV, figs 17 and 18) or the bulge may be inwards causing an indentation of the muscle substance, although this form is less usual (Plate XXXV, fig 19)   The cytoplasm of the containing muscle-sheath cell is represented by a granular debris occupying the spaces between the parasites   This is probably purely a fixation effect as the appearance of the nucleus suggests that the cell is not appreciably damaged by the parasitic invasion

A third form of distortion occasionally seen simulates a pedunculated cell (Plate XXXV, fig 20)   This, however, is not an indication of an actual pedunculated structure and is due to the plane of the section passing so as to cut through

the parasitized cell and exclude part of the corresponding muscle   This is evidenced by studying such examples in serial sections

(*f*) *Appearances seen when the parasite is in the substance of the muscle* —The appearances represented here vary according to whether the muscle is examined in cross or longitudinal section   In the former case the parasites are seen to be lying in greater or smaller aggregations in spaces within the muscle   There is no regular shape in these aggregations and there appears to be no particular cellular reaction on the part of the muscle to shut off these groups from the muscle-fibres (Plate XXXIV, fig 15)

In certain cases the section of muscle appears to be somewhat more granular than is normally the case and there is some indication of a fibrous or fabrillar structure   This is indicated in Plate XXXIV, fig 15, but is not always apparent and may be due to minor variations in the technique of fixation

In longitudinal sections of muscle the parasites are found disposed in long lines separating the muscle-fibres (Plate XXXV, fig 21)   These lines of parasites may be several parasites thick but we think the tendency is rather to form sheets of parasites than columns   By this we mean that once the parasite has established itself between the muscle-fibres it tends to multiply laterally in sheets as being the line of least resistance rather than to separate still further the muscle-fibres in order to develop in a depth of several layers of cells   That this is not always the case, however, will be seen from Plate XXXIV, fig 15

Occasionally the beginning of the processes here described may be seen in the form of a single parasite embedded in muscle   Such a form in a process of division is shown in Plate XXXV, fig 22   The form of the parasite occupying the muscles and the sheaths must now be considered

In the previous Section it was shown that after the development in the pseudocysts of the club-shaped bodies the latter, either by active penetration of the cell wall or by its rupture, left the pseudocysts and actively migrated into the tissues of the host

Here they may, rarely, be seen although their scattering renders this difficult except by the most laborious search of many sections   We presume, however, that their migrations, except in abnormal circumstances, do not last for long and that they soon reach their objective in the muscle or its sheath   It is presumed that in this case the penetration of the cell is an active process on the part of the parasite, endowed as it is with motility while the muscle or sheath cell is fixed

We have, on more than one occasion, seen the proof of this initial invasion, as evidenced by the presence of one or two large parasites in cells of the muscle-sheath We have even seen two club-shaped forms occupying such a situation   As there is no evidence that the club-shaped bodies, while still retaining their club-shaped form, undergo division nor that they exhibit any sexually differentiated forms we presume that this represented the invasion of one cell by two independent and similar club-shaped forms

Having gained entrance to a cell of the muscle-sheath the club-shaped body rounds up into comparatively large subovate bodies and the chromatin of the nucleus commences to fragment in what appears to be a rather irregular manner (Plate XXXV, figs 23 and 24)

This is the commencement of a process of multiplication This process does not appear to be one of true schizogony since the subdivision of each parasite does not proceed to the formation of more than a few daughter bodies It seems rather to be the result of precocious equal divisions of the nucleus while the cytoplasm lags in the process Each such dividing group eventually forms a limited number of smaller parasites and from the degree of invasion of the muscles these would appear to repeat the process Various stages in this process are shown in Plate XXXV, fig 21

In certain cases all the parasites appear to be relatively uniform in size and are chiefly unnucleated (Plate XXXIV, fig 18) In such cases it would appear that division had come to an end and the parasites had assumed a resting phase

That this is probably the case is shown by the fact that no further extension of the infection to other tissues or organs of the adult tick has been noted and one has been forced to the conclusion that the invasion of the muscles by the parasites and their multiplication in this situation constitute the end of development so long as the adult tick remains unfed The course of events when such an infected tick feeds will be described in the next Section

At this point it may be convenient to recall the discussion at an earlier stage of the nature and functions of the cells which enclose the parasite in its earlier stages These cells were considered to be wandering phagocytic cells The cells of the muscle-sheaths which we have now been considering must also be considered phagocytic inasmuch as they also, actively or passively, engulf the parasites The rôle played by the earlier wandering phagocytic cells and that played later by the fixed phagocytic cells of the muscle-sheaths would appear to suggest comparison with the wandering and fixed cells of the reticulo-endothelial system of vertebrates and it is difficult to avoid the conclusion that, in actual fact, the function of these cells in the tick is actually analogous to the cells of the reticulo-endothelial system of vertebrates Dr Wigglesworth of the London School of Tropical Medicine, to whom the preparations of microscopical sections were shown, was of opinion that the phagocytosing cells were blood cells of the tick and it would appear that this strengthens the contention that their function may be aptly compared with that of wandering and fixed cells of a reticulo-endothelial system

(g) *Appearances shown by* B canis *after the adult, developed from the infected nymph, has commenced its blood meal on a dog —*The investigation of this part of the life-cycle of *B canis* has proved very arduous on account of technical difficulties The number of ticks which, as adults, remain infected out of any batch of nymphs fed on an infected dog, is comparatively very small This, added to the fact that, throughout this inquiry, the task of elucidating each point by the examination of sections of whole ticks in which all the tissues and organs are left intact and *in situ*, was voluntarily accepted has rendered necessary the study of a vast amount of material The result has been that, while the main points of the total inquiry have been fully worked out, full information with respect to the stages intervening between the stage when the adult tick, heavily parasitized in its muscles, commences feeding and the stage where it passes on the infection by its bite to a new dog host has not yet been acquired

When a tick is put on to a dog it does not at once start feeding but at first wanders about seeking a suitable place to attach itself Even when it becomes

attached it does not immediately commence to feed so that after the tick has been placed on its host it is probably usual for about two days to elapse before the tick commences its blood meal   The actual repletion of the tick also takes a long time so that an adult tick may sometimes remain attached for as long as twelve days At the end of three days after attachment of the tick feeding will definitely have commenced and blood will be found in some of the gut diverticula   It seems probable that the digestive process whether carried on by secretions or by digestive cells, commences very soon after the tick is attached, at least in the form of a preliminary mobilization of cells or secretion or both, because the blood meal even at this early stage shows signs of active digestion and it is unusual to be able to recognize the individual red cells   Rather the blood meal has the appearance of a pinkish amorphous mass in which the white cells, being more resistant, are distributed as more darkly stained masses

The condition will now be described as met with in an infected adult tick three days after its attachment to a dog

At this stage in the development of the tick the salivary glands constitute a large part of the total contents of the tick and extend on each side of the middle line from near the anterior to near the posterior end (Plate XXXIX, fig   25) Structurally they consist of a wide-lumened chitinized central duct on each side from which branch off subsidiary chitinized ducts which, again, branch into smaller ductules   These ducts bear away the secretion from a very large number of simple acini consisting of a single layer of cells surrounding a lumen with their bases resting on a basement membrane   In section the outline of the acinus is more or less circular and the cells are large and shaped like low truncated pyramids   In the cells are contained large masses of zymogen granules which stain pink with Giemsa's stain and render the salivary glands striking objects under the microscope

Among the ordinary salivary acini, are seen certain very large acini which do not contain the characteristic zymogen granules   These acini are probably those described by Christophers (1907) as ' poison acini '   These acini tend to be larger in size than the ordinary secreting acini and, while they presumably secrete some fluid product, this does not seem to have any easily visible precursor in the cells in the form of the zymogen granules which are such a striking feature in the ordinary salivary acini

In infected ticks either the poison or the salivary acini may contain parasites and the appearances seen will be described separately

The acini usually found infected are the ordinary salivary acini and it seems to be usual for these only to be infected   In the cases where the poison acini have been found infected—only two or three in a fairly large series—the ordinary salivary acini in the same ticks were free of infection   Whether this is the general rule, i e , that only one or the other kind of acinus is infected and never both in the same tick is not certain but such was the case in all the infected ticks examined *

The description will first be given of the infected poison acini as these, although much less common, were actually the first forms found in salivary gland infections in this study   The appearances seen in the ordinary salivary acini will then be described and compared with the former

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* A case where both kinds of acini were infected has subsequently been seen,

Appearances seen in the poison acini (Plate XXXVI, fig 26)—In infected ticks these acini may be enormously enlarged so as to extend to three times the diameter of the ordinary salivary acini   They consist of a single layer of cells more or less like low truncated pyramids surrounding a comparatively large lumen

These cells are found to be heavily parasitized with *B canis* and this parasitization is so intense in some cases that the whole acinus seems to be almost uniformly filled with the parasites and the lumen is almost completely occluded

The parasites at this stage are characteristic in appearance and differ from the forms previously described as resting in the muscles

The individuals tend to be spherical or ovoid in shape and have a very large mass of chromatin which is situated eccentrically and sometimes occupies half the body of the parasite   Active multiplication is in progress and this appears to be by a process of binary fission   Where division is especially active the parasites are found clumped together in greater or lesser groups giving a ' morula ' or raspberry effect   At this stage the limits of the gland cells are still visible, although somewhat obscured, and the lumen of the acinus when present is either clear or filled with granular debris   In other cases the lumen may be absent, the cells all meeting in the centre of the acinus   As the cells of the acinus become congested with parasites there is presumably rupture of their walls with the setting free of parasites into the lumen   Eventually the whole structure of the acinus may be almost completely replaced by a mass of parasites but the nuclei of the acinar cells do not appear to be much affected and stain well up to a late stage in this process   Eventually, however, they seem to fragment and stain less distinctly

The cytoplasm of the gland cells consists of an amorphous coagulated granular debris   There may be a fairly large and patent lumen in the acinus or the cells may converge towards the centre so as nearly to occlude it   In the lumen, when present, there is often some amorphous debris which presumably represents the coagulated secretion of the acinus

By the time this condition of intense multiplication has been reached the individual parasites, in some areas packed closely against each other, are somewhat smaller in size than when the infection of the poison acinus was at an earlier stage but they retain the same globular or ovoid form previously described   This appears to be the infective form as no further modification has been found even in infections at the latest stage examined and where the ticks have already actually infected dogs by their bite and, therefore must have contained the actual infective forms

The condition will now be described which is met with in an infected adult tick nine days or more after its attachment to a dog

The appearances shown by the salivary gland in general have been dealt with in a previous Section and the conditions found when the infection with *B canis* is confined to the poison acini have been described   In this Section, therefore, the appearances presented when the infection is present in the ordinary salivary acini will be especially considered

Appearances seen in the ordinary salivary acini (Plate XXXVIII, fig 43)—The general appearances shown by these acini in a normal tick have previously been briefly described and need not be further detailed here   In one and the same tick all degrees of infection may be seen in different acini, from those in which but a single

cell is infected and that with only a few parasites, to those whose cellular structure is almost completely replaced by a massive growth of parasites filling nearly all the available space within the limits of the basement membrane of the acinus (Plate XXXIX, fig 27a) In the intermediate condition between these extremes the acinus may show one or two cells heavily parasitized, while the remainder are normal or may contain only a few parasites (Plate XXXIX, fig 27b) The normal cells of an infected acinus and those containing only a few parasites appear to be physiologically active and produce zymogen granules (Plate XXXIX, fig 27c) but those cells which are heavily parasitized have their volume completely occupied by parasites which renders impossible the production of the normal secretion The heavily infected acini are definitely increased in size but never reach the dimensions sometimes reached by infected ' poison ' acini

The parasites in infected cells show a tendency to be very closely crowded together, more so than in the case of the infected ' poison ' acini, forming compact parasitic masses within the cytoplasm of the infected cell or completely replacing it The periphery of these parasitic masses is usually clear cut and regular in outline (Plate XXXIX, fig 27a and fig 27c), the increase in numbers of parasites showing no tendency to take the form of a break away from the parent mass This characteristic of solid masses of parasites with regular outline is again more characteristic of the invasion of the ordinary salivary acini than of the poison acini where the growth tends to be less compact on the whole

When the acini of the salivary glands show the extreme degree of parasitization sometimes encountered one would expect to see the secretory ducts containing large numbers of parasites in process of expulsion towards the proboscis This, however, is not the case and although a certain number of undoubted parasites may be seen in the ducts they are comparatively rare This is doubtless due to the fact that the tick may remain attached to the host for over 12 days and probably only feeds at intervals It seems likely that the parasites are transferred to the dog host chiefly during the actual acts of feeding or possibly just previous to them and that only specimens removed at the appropriate time, which there is no means of knowing, would show parasites in numbers in the ducts

In ticks which have remained attached for 12 days or more to their host the salivary glands have a characteristic exhausted appearance The acini, instead of being lined with truncated pyramidal cells enclosing a small lumen, are now lined with comparatively flattened cells enclosing a large lumen and containing practically no zymogen granules (Plate XXXIX, fig 28) In infected ticks at this stage it is not unusual to find the majority of the acini in this condition while a few infected acini still retain the rounded plump condition lined with truncated pyramidal cells containing both parasites and zymogen granules

In the ordinary salivary acini the final form of the parasite appears, as in the case of the poison acini, to be globular or ovoid This is not always easy to determine in the case of the solid parasitic masses but where individuals, or less closely aggregated groups, are seen a globular or ovoid form seems to predominate Exceptions to the rule are probably mostly due to parasites being distorted by compression, cut tangentially, or in process of division In certain masses somewhat smaller and elongated forms have been noticed but no special significance has been attached to them.

(B) *If ingested from infected dog's blood by the adult stage of the dog-tick,*
R sanguineus —It has already been stated that most of the observations in this
study on the life-history of *B canis* in the dog-tick have been made on the sequence
of events following the ingestion of infected dog's blood by the nymphal stage of
the tick   In this Section, therefore, only those points will be considered which have
come under actual observation as the result of feeding adult ticks on infected dog's
blood and no attempt will be made to follow up the complete sequence of events as
was done in the case of ticks primarily infected as nymphs   It is obvious that the
sequence must be different, at least at some stages, since in the case of the tick fed
as a nymph the end product, on transformation of the nymph into the adult, is an
accumulation of parasites in the muscles and muscle-sheaths, while in the case of
the tick fed as an adult the end product is an accumulation of parasites in the ova
leading to persistence of infection in the next generation of the ticks   It should be
mentioned here that, although both male and female adult ticks were fed by us, only
the latter were systematically examined in connection with this investigation

The appearances shown by *B canis* in the early stages of its development
after ingestion by the adult tick have not been investigated   The most detailed of
recent accounts, that of Regendanz and Reichenow, indicate that the early stages
of development, once the parasites have left the lumen of the tick's gut, occur in the
cells of the gut wall   Here a single individual multiplies by binary fission to produce
an immense number of small forms which are destined to infect the eggs of the
tick for the transference of the infection to the next generation   This development
in the gut epithelium is certainly not found in the case of the nymphs of *R sanguineus*
and the present observations in adults, so far as they go, have given no indication
of any such localization but, in the absence of any positive findings, one does not
care to be dogmatic

Dennis (*loc cit*) in describing the development of *B bigemina* in *Margaropus
annulatus* does not describe any development in the gut epithelium of the adult
tick but his description of the actual development of *B bigemina* is short and
difficult to follow and some of the diagrams depict stages to which the preparations
used in this study show no counterpart

We may leave, therefore, the elucidation of the early stages of development
in the adult and proceed to the description of findings in the stages during and
after penetration of the ova by the forms of the parasite destined to produce here-
ditary infection in the next generation of ticks   As soon as the adult tick commences
feeding there is a rapid development of the ovaries   These consist of a single-tubed
organ arranged roughly in the form of a U- from the two anterior ends of which there
pass forward the oviducts   With the continuation of the tick's meal the ova nearest
the main duct rapidly enlarge and acquire a chitinous covering and this process
continues until the tick's meal is completed after twelve days or more   In such
fully-fed ticks it is almost impossible to obtain really satisfactory sections of the
whole tick   This is due to the fact that the digested blood meals, taken over twelve
days or more, have been reduced to a granular amorphous dark-brown semi-
crystalline looking material which is probably altered hæmoglobin and which it
has been found impossible satisfactorily to fix, no matter what fixative solution is
used   The reason for this is probably that the material contains no albuminous
or other matter capable of coagulation by any fixative   This unfixed material,
which occupies the largest part of the entire bulk of the tick, will not cut

satisfactorily and also interferes with the cutting of the other tissues which may be distributed around or between the diverticula of the gut   In certain cases, however, when conveniently situated, these tissues are satisfactorily cut and can be studied   The remarks made above with regard to the fixation and cutting of the blood meal must be understood to apply only to the digested meal   Freshly ingested blood fixes, cuts and stains quite satisfactorily   If the ovaries or other organs are removed from the ticks and independently fixed and cut they present no particular difficulties but, as previously stated, an endeavour was made wherever possible, to avoid this and to cut whole ticks in order to preserve intact the relations of all the organs studied   With very few exceptions—only in certain cases in the ovaries—all the observations have been made on such whole ticks

If the ovaries are examined in a section of an adult female tick when fully fed it will be found that the ova are present in all stages from mature ova covered with a chitinous covering to the smallest immature ova   It will be found that the contents of the younger ova are perfectly fixed but that those of the older ova with chitinous covering are less so and are fragmented into irregular masses   In spite of this lack of proper fixation in the older chitin-covered ova it was often observed that parasites contained in these ova were quite well preserved and could be satisfactorily studied

The forms of parasites encountered when adult ticks were fed on infected dogs will now be described but, as the changes occurring in the infected adults have not been studied in the same detail as when the primary infection occurs in nymphs, one is prepared to find that attempts to correlate the forms of *B canis* seen, as regards the order of their occurrence, may need revision as the result of more exact study   If the accounts given by Regendanz and Reichenow and by Dennis are correct the club-shaped forms to be described are the forms which migrate from the site of their preliminary development in the gut cells or elsewhere to the ovaries where they penetrate the ova   From the fact that in the writer's preparations these forms are sometimes found in mature ova while the smaller forms, developing from the ' clubs ', are also found in immature ova it would appear either that the club-shaped forms are able to penetrate ova after they have received their chitinous covering or that, after penetration of a less mature ovum, they do not necessarily go on to develop into the smaller forms, or at least exhibit a delay in doing so

(a) *Club-shaped forms* —These forms are the ones mentioned by Christophers who gives a description of their formation from rounded forms of parasite   The method of their formation in the nymphal stage of the tick has already been described in this paper   They have been met with inside mature ova lying closely under the chitinous covering   In this situation it is probable that they are at first motile because they may be found either straight (Plate XXXVII, fig 29) or doubled upon themselves (Plate XXXVII, fig 30)   Owing to the comparatively large size of the club-shaped forms they are not always complete in a thin section and one of the forms illustrated in Plate XXXVII, fig 29, is probably either not a complete club or is fore-shortened

(b) *Forms of irregular shape but tending to be ovoid when units* —These forms vary greatly in size and are to be found apparently both in the ova and in the follicles which contain the latter   It seems probable that the parasites in the follicles represent forms which have failed for one reason or another to penetrate the ova and have continued their development in the follicle.

1 *Mononucleate or binucleate large forms* (Plate XXXVII, figs 31, 32 and 33) —These forms are of various shapes and sizes and probably represent club-shaped forms which have lost their characteristic shape and in which division into smaller forms has been initiated

2 *Multinucleate large forms* —These forms also vary much in size and shape (Plate XXXVII, figs 31, 33 and 34) and represent the multiplication of the previous forms carried a stage further   In calling these forms multinucleate one does not mean to infer that they represent a condition of schizogony, although that is the appearance sometimes presented   Rather one considers that the multiplication is by a process of binary fission in which the division of the cytoplasm sometimes lags behind that of the nucleus

3 *Mononucleate small forms* —These appear to represent the end stage of development in the ovum   They are ovoid, contain one compact nucleus and are not more than one-third the diameter of the larger forms previously described as occurring in the ova (Plate XXXVII, figs 35 and 36)   These forms occur both in the ova and in follicles from which the latter have been discharged   There appears to be a distinct tendency for these forms to be aggregated in the peripheral part of the ovum, i e , lying closely under the chitinous coating of the ovum but this is not an invariable rule   Occasionally two forms may be found lying together in a cyst like space (Plate XXXVII, fig 37)

At this stage one may draw attention to Plate XXXVII, figs 38 and 39, in which are depicted various forms of *B canis* met with in ordinary ' squash ' preparations made by smearing out a number of eggs between two slides   It will be noticed that some of the forms are very irregular while others are exceptionally large   This is due to the method of making the preparation and such results justify the attempt to use only material fixed in the tissues for our descriptions   The irregularly ' spiked ' forms are similar to those described by other authors and, in the writer's opinion, are probably largely artificial although some rather angular forms are also met with in sections (Plate XXXVII, figs 31 and 33)   The smear preparations would represent forms such as might be met with if one tried to represent the shape of the contents of a bird's egg from the shape they assumed after dropping on the floor

In describing the sequence of events when the nymphal stage of the tick fed on infected blood the description of the development of *B canis* in the nymph was carried up to its conclusion in that stage of the tick and then its further development was followed up in the next stage, the adult   To preserve the same principle one may now consider the appearances shown by the parasite in the larval forms of the tick which hatch out of the infected eggs

Appearances shown by B canis *in larval ticks hatched from infected ova* —The forms found as a hereditary infection in larvæ have only been studied here in so far as the appearances seen in unfed larvæ hatched about seven days are concerned   The method of making sections of large numbers of such larvæ has been described in the Section on technique   This method, by rendering possible the simultaneous examination of large numbers of larvæ, enables one quickly to decide whether a large or small proportion of the larvæ is infected, as the entire larvæ can be readily followed from section to section when these are mounted serially   Such examinations have shown that when the parent tick is heavily infected a very large

proportion of the larvæ hatched from its eggs are infected   Actual counts have not been done to determine the percentage but in some cases at least fifty per cent of the larvæ show infection with *B canis*   This does not, of course, imply that all these larvæ are *ipso facto* capable of transmitting infection or even of handing it on to the next nymphal stage, any more than that all nymphs fed on a heavily infected dog and, therefore, necessarily ingesting large numbers of parasites, are capable of developing into infected adults—it is known that a comparatively small proportion do

The final stage of *B canis* in the ovum has already been described as a small uninucleate form and in unfed larvæ forms which correspond in every way with these are the only ones met with (Plate XXXVII, figs 40 and 41)   This gives one reason to believe that, as in the case of the infected adult, a migration of the resting forms to the glands preliminary to active transmission may only occur after the larva begins to feed   This is taking it for granted that the larva transmits the disease by its bite as in the case of the adult   There is one theoretical objection to this assumption, viz , that the larvæ only remain attached for three to five days which gives a very short time for migration of parasites to the salivary glands and their multiplication there to produce infective forms, compared with the 12 or more days which the adult remains attached   As will be seen in a later Section the writer has succeeded in infecting dogs by putting on them infected larvæ but this does not wholly eliminate the possibility of contaminative infection of the dog via the alimentary tract if such a method is possible, a point we have not yet tested

In sections of larvæ which have recently hatched from eggs and not yet fed it will be found that the body of the larvæ still contains considerable quantities of undifferentiated material which is taken to be the still unutilized yolk carried over from the ovum stage   This condition persists for at least a week and it is in this residual yolk that the forms of *B canis* found in the larva occur   These forms are small uninucleated spherical or ovoid bodies indistinguishable from the small uninucleate bodies described as the final stage of *B canis* in the ovum   They are fairly uniform in size and are always found in groups sometimes of considerable size numbering well over a hundred individuals (Plate XXXVII, figs 40 and 41) Their further development and history have not been investigated except in so far as that the infectivity of the larvæ has been demonstrated

(C) *If ingested from infected dog's blood by the larval stage of the dog-tick,* R sanguineus —Owing to lack of time the sequence of events following the ingestion of infected dog's blood by larval ticks has been only tentatively investigated from the point of view of the morphology of the parasite and one does not feel sufficiently certain of the few observations made to record them   The experimental evidence obtained that these larval ticks become infected by feeding and infective in the next stage has already been noted and will be mentioned in the Section dealing with the life-cycle of the parasite

VI   LIFE-CYCLE OF *B CANIS* IN *R SANGUINEUS*

The various stages in the life-cycle of *B canis* in *R sanguineus* have been dealt with *seriatim* but necessarily somewhat disjointedly in the descriptions given in Sections above but, for the sake of clarity, these part descriptions may now be brought together and welded into a whole as coherent as the missing portions will allow

It will have been evident from the Sections preceding this that only in the cases of ticks fed as nymphs has the life-history of *B canis*, with the exception of the earliest stages, been completely worked out and it is only the life-cycle, as elucidated when it originated in the nymphal stage, which it is proposed to set forth here   The fragmentary information relating to events when the adult or larval ticks are the stage infected from the dog may be sought in the relative sections, the information being too meagre to weld into a life-cycle

Another point which should be mentioned here is that the time-periods mentioned for different stages, both of the parasite and of the tick, as also for the feeding and attachment times of the latter are those holding in the cool climate of Kasauli   The periods would be considerably and, in some cases greatly, diminished in a warm climate such as that of Madras

Life-cycle of B canis *in* R sanguineus *when the nymphal stage of the latter sucks the blood of an infected dog* —Christophers, Koch, Regendanz and Reichenow, Dennis and others have described the earliest forms met with in the gut of the tick when fed upon various species of *Babesia* and the papers quoted in the list of references may be consulted for their descriptions   In the present study, which has been, almost entirely, a study of whole ticks fixed and cut, no evidence of any stages such as the typical ' club ' forms found at a later stage, but which some workers describe as occurring in the gut, has been come across in the early stages of infection   Neither has any evidence of the coalescence of sexually differentiated parasites been obtained   In freshly ingested blood forms, unaltered in appearance from those seen in blood films, may be seen, sometimes still enclosed in blood corpuscles, but more often lying free in either case there is evidently a great destruction of parasites in the gut of the tick and the survivors, compared with the numbers in the ingested blood, are comparatively few   These free parasites are sometimes found clumped into groups, whether fortuitously or otherwise cannot be stated   Whatever the course of events may be in the lumen of the gut of the tick the first definite and unequivocal evidences of development of the parasites noted in the writer's sections of nymphs were in nymphs four or five days after they had completed their blood meal on an infected dog and dropped off the host

At this stage *B canis* occurs as a globular body with a single small nucleus and it is found in a large phagocytic cell usually situated closely under what has been called the epidermal layer of cells seen at this stage lying under the outer chitinous covering   In the absence of any evidence as to whether this stage is the result of the union of sexually differentiated parasites, or otherwise, it is difficult to give it a name and we may, therefore, tentatively call it the ' pseudo-zygote '

The nucleus of the pseudo-zygote becomes more bulky and divides into two Each nucleus re-divides until eventually the parasitic body, now greatly increased in size, becomes multinucleate, some of the nuclei apparently being connected together by threads of chromatin   Two such parasites may not infrequently be found in one cell, and they may be at different stages in the process of development to he described

When the subdivision of the chromatin has resulted in a large number of nuclei the cytoplasm of the pseudo-zygote condenses around each small mass of chromatin to form a globular or ovoid body and this results in the complete transformation of the pseudo-zygote into a clump of such bodies contained within the envelope of

the parasitized phagocytic cell   The whole structure so formed and including the containing cell strongly resembles a cyst and may, for convenience, be referred to as the 'pseudocyst'   These pseudocysts are sometimes sufficiently numerous themselves to form small groups of three or four usually lying closely under the epidermal layer previously referred to   About seven days after the nymphal stage of the tick has left its host the pseudocysts are fully developed and one may contain as many as two hundred individual parasites   These parasites may, for convenience of description, be termed primary sporoblasts, having arisen by division from the pseudo-zygote   The next development in the life-history, about eight to ten days after the nymph has left its host, is the development of each of the primary sporoblasts into a club-shaped body   This is initiated by the appearance of an achromatic line across the parasite which, if Christophers' description is correct, partially divides the parasite into larger and smaller portions, the smaller and narrower part swinging round to form a club-shaped body   These lines may be seen present in practically every parasite in the pseudocyst if examined at the correct stage   When the development of the primary sporoblasts into 'clubs' is completed the latter may still be contained within the pseudocyst   This stage is reached eleven to fifteen days after the nymph has left its host

This development is a provision for eventual migration of the parasite from the pseudocyst and this migration now takes place   The line of migration will evidently vary with the location of the pseudocyst and the club-shaped bodies have been met with as individuals in different tissues but their objective appears to be definite, viz, the muscles and muscle-sheaths

This invasion of the muscles is first evidenced by the finding of occasional club-shaped forms or the latter, somewhat rounded up, in cells of the muscle-sheaths which they appear to have recently penetrated   On occasion two such bodies have been noted in a single cell of a muscle-sheath   The clubs, having reached the site of election in the muscles or muscle-sheaths round up and commence to divide and the individuals so formed may be termed the secondary sporoblasts   The result of the division depends to a large extent on the situation of the invading forms   If in a cell of the muscle-sheath they may form a large number of parasites which form a bulge on the external surface of the muscle or they may spread more thinly over its surface   If the original migrants get between the muscle-fibres they may multiply either in sheets or in long lines along the planes between the muscle-fibres   In either case the parasites, originally relatively large tend, in the process of division, to become smaller   This process appears finally to reach a static condition but, before the time this stage is reached, the nymph has changed into an adult, the period that has elapsed since the feed of the nymph being about 20 days or more   In the adult, once the repeated division of the parasites is completed, no further change appears to take place and the condition of infiltration of the muscles with parasites which have been called the secondary sporoblasts remains until the tick begins to feed   It should be pointed out here that, although this invasion of muscles may be found in any situation where muscle exists, it is not usual for all the muscles to be affected   Usually some groups only are infected, and sometimes only those of one side may be parasitized   The infection, so far as observations to date go, appears to be confined to the muscles of the body proper

The adult tick, in the ordinary course of events, now commences to feed on a dog   This act appears to initiate a migration of the parasites located in the muscles

towards the salivary glands of the tick   What the stimulus is which causes this migration is not known but, from the wide dispersal of the parasites in the muscular tissues, it is presumed it must in some way be conveyed in the circulating tissue-fluids   The mechanism by which the parasites transfer themselves from the muscles to the salivary glands is also not known as they have not been observed in the process of migration   The process, however, must be an active migration by motile forms since the parasites are found in the interior of muscles and muscle-sheaths and, arguing by analogy from the motile forms in earlier stages of the life-history, it is reasonable to suppose that these will resemble the club-shaped forms which served a similar purpose earlier in the life-history   In the absence, however, of direct observation of such forms it is necessary to postulate the alternative possibility that the migration may be accomplished by amoeboid movement of the muscle forms, although this seems less likely   The fact that Cowdry and Ham (*loc cit*) have recorded the finding of club-shaped forms in the salivary glands would appear to indicate the former hypothesis as the more probable

However the migration is accomplished, within two or three days of the tick becoming attached the salivary glands have already a well-established infection so that the process of invasion must be a rapid one   The secondary sporoblasts on reaching the ordinary, or possibly the poison, acini of the salivary glands immediately initiate a process of intensely active multiplication resulting in the packing of the infected cells, and sometimes of the whole acinus, with a mass of smaller rounded or polyhedral forms which at last may be so closely applied to one another as only to be recognizable as individuals by the single darkly-staining nucleus included in each   These final forms are the infective sporozoites   In such heavily infected acini the structure of the secreting cells is destroyed and the parasites invade the lumen of the acinus, are carried along the salivary ducts and injected into the tissues of the mammalian host during the feeding acts of the tick as long as the latter is attached to the host   This completes the life-cycle of *B canis* in its arthropod host *R sanguineus* when infective blood is ingested by the nymphal stage of the latter

Methods of infection of dogs by infective ticks—To round up the description of the life-history of *B canis* in *R sanguineus* a short account may here be given of the different methods in which infection may be conveyed to the dog   These methods come under two heads which will be considered below —

HEREDITARY INFECTION —In this case the form of the tick to acquire the infection is always the adult   The infection persists through the egg stage and is transmitted by the larva or nymph or both   It is believed that, until demonstrated in the course of this inquiry, infection through the larval stage had not previously been proved   The infection is definitely by bite in the case of the nymph and probably so in that of the larva

STAGE TO STAGE INFECTION —The probability of this was envisaged by Christophers but has been definitely proved experimentally in the course of this inquiry By stage to stage infection is meant the fact that whatever stage of the tick acquires the infection the succeeding stage is capable of transmitting it   Thus, if the adult acquires the infection by feeding on an infected dog the larva can transmit it to a healthy dog , if the larva acquires the infection the nymph can transmit it and if the nymph acquires the infection the adult can transmit it   All these combinations have been experimentally proved in the present investigation

Part II.

CYTOLOGY OF THE DIFFERENT STAGES IN THE LIFE-CYCLE OF *B. CANIS* IN *R. SANGUINEUS.*

These stages will, for convenience, be taken in the order in which they have already been dealt with and a short description of each be given where differences in detail warrant a special description   The descriptions given are from the appearances seen in sections fixed in Bles' solution and stained with iron hæmatoxylin

Cytology of the forms of B canis *seen in the nymph of* R sanguineus *four or five days after it has completed its meal and dropped off the infected dog*

These forms consist of the early stage of *B canis* before it has divided up to form primary sporoblasts   They are contained in the wandering phagocytic cells already alluded to

*Shape —*The shape of the parasite at this stage tends to be spherical at first and with a regular contour, as is seen in the smallest forms depicted (Plate XXXII, fig 3)   As the parasite grows in size the spherical form may be modified into various ovoid forms due to adjustment of the contour to neighbouring structures   This is especially evident where two or more forms are present in one cell (Plate XXXII, fig 6)   Such forms retain their regular contour, by which is implied the fact that there are no sharp angles in the contour

*Size —*The smallest forms encountered at this stage measured 3 5μ in diameter Much larger forms may be encountered in which the fragmentation of the chromatin is well advanced but which do not as yet show aggregation of the cytoplasm to form primary sporoblasts   Such forms may measure 9μ in their longest diameter (Plate XXXII, fig 4)

*Cytoplasm —*The cytoplasm of these forms has a uniform finely granular appearance resembling ground-glass and a general freedom from cytoplasmic inclusions other than nuclear chromatin   There is also usually an absence of any vacuolation   The cytoplasmic body appears to be surrounded by a distinct cell membrane but there is no evidence of division of the cytoplasm into zones of ectoplasm and endoplasm

*Nucleus —*In the earliest stage the nucleus is a single spherical dot of chromatin (Plate XXXII, fig 3) sometimes surrounded by a slight halo simulating rarefaction of the cytoplasm   The appearances shown by the nucleus in the further evolution of this stage of the parasite will be most conveniently described under ' division ' in the next Section

*Division —*The initial division of the earliest form of the parasite at this stage is indicated by an increase in bulk of the nucleus followed by its elongation (Plate XXXII, fig 3)   The elongated nucleus is surrounded by an elongated halo

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in the cytoplasm   The process appears to be a simple amitotic division   We are not certain whether the process just described leads to the division of the parasite into two individuals or, as appears more likely, is the first stage in the fragmentation of the chromatin to be now described   This process consists in repeated division of the chromatin into larger or smaller masses until it is widely distributed in the cytoplasm (Plate XXXII, figs 4, 5 and 6)   These masses sometimes appear to be connected together by threads of chromatin but this appearance is less evident than it is in the case of smear preparations where the long threads of chromatin are, partly at least, artefacts   This process of fragmentation of the chromatin is accompanied by a great increase in the general bulk of the parasite as will be seen by comparing Plate XXXII, figs 3 to 6, all of which are drawn to the same scale This stage is followed by an evening up of the size of the fragments of chromatin accompanied by a gradual condensation of cytoplasm round each mass to form the primary sporoblasts   This stage is depicted in Plate XXXII, fig 7, which shows commencing condensation of the cytoplasm

Effect on parasitized cell—Apart from the actual mechanical occupation of its cytoplasm the parasitized cell does not appear to be greatly affected, as evidence of which its nucleus stains normally   In certain cases, however, the cytoplasm is seen to be closely packed with globules, somewhat resembling secretory globules These globules vary in size in different cells but are usually of approximately the same size in any one cell (Plate XXXII, figs 4, 5 and 6)   Their nature is not known

Cytology of the forms of B canis *seen in the nymph of* R sanguineus *about seven days after it has completed its meal and dropped off the infected dog*

This is typically the stage of pseudocysts containing the primary sporoblasts The pseudocysts as a whole have already been described in a previous section and may be dismissed with the observation that they vary greatly in shape, for reasons already given, and in size   They may vary from 14μ to 35μ in greatest diameter and their size does not necessarily bear any relation to the state of development reached by the contained primary sporoblasts   The latter may be considered under two stages of development, (*a*) primary sporoblasts and (*b*) pre-club forms

(*a*) *Primary sporoblasts*

Shape—Each primary sporoblast is a spherical or ovoid body with regular contour   When closely applied to one another the contour may be somewhat less regular but is never angular

Size—The size of the primary sporoblasts varies from $1\,7\mu$ to $3\,3\mu$ in the longest diameter but it is usual for all the individuals in any single pseudocyst to be approximately the same size   On the other hand the primary sporoblasts in contiguous pseudocysts may vary considerably in size

Cytoplasm—The cytoplasm has throughout a uniform ground-glass appearance and is free from inclusions other than the nucleus   No vacuoles are present Surrounding the cytoplasm there is a delicate cell membrane

Nucleus —The nucleus tends to be a single small compact mass of chromatin in the majority of the forms and is too small to enable any structural details to be distinguished   In certain cases, in place of the small compact mass the nucleus may be lengthened out or be somewhat irregular in shape (Plate XXXII, fig 9) This might be interpreted as a stage in division but we are not satisfied that it is so as we are not quite sure whether there is ever actual division of the primary sporoblasts in the pseudocysts once there has appeared the condensation of cytoplasm round the fragmented chromatin as described in an earlier section   Were such division in the primary sporoblasts common there should be more evidence of it in the numerous pseudocysts which are sometimes to be seen in the nymph

Division —This question has been dealt with in considering the nucleus

Effect on cell —In all cases the cell containing the primary sporoblasts, and forming the wall of the pseudocyst, is distended by their presence and its own cytoplasm is, for all practical purposes, replaced by the primary sporoblasts   Its nucleus is always pushed to the periphery of the pseudocyst but its actual appearance with respect to the latter will depend on the orientation   It may be centrally placed or quite peripheral or it may be completely masked   All these forms are to be seen in Plate XXXIII, fig 10

(b) *Pre-club forms*

Shape —The general shape of these forms is that of an ovoid with tags or excrescences varying in shape with the stage of development reached by the individual form   The general contour is regular but is interrupted by a loss of continuity at one or more points where the achromatic lines to be described under ' cytoplasm ' cut the periphery of the parasite   In some cases tags, appearing like sections cut off the ovoid, are lying in juxtaposition to the main portion of the parasite and separated from it by the achromatic line   In other cases the achromatic line does not reach the periphery at one end and then the main portion of the parasite appears to have a short, stout re-curved tail

Size —The size of the pre-club forms is fairly uniform varying between 2 5 and 4μ in the longest diameter

Cytoplasm —The cytoplasm has a uniform ground-glass appearance somewhat rougher in effect than that of the primary sporoblasts and stains well   There are no cytoplasmic inclusions or vacuoles but, traversing it in a direction usually such as to cut off a section of the parasite, is an achromatic line   The line does not actually cut off a section as it often appears to do, this effect being produced by the orientation of the parasite and the way in which the section has passed through it In the larger of the two parts into which the parasite is divided by the achromatic line lies the nucleus   Surrounding it there is a distinct halo of apparently rarefied cytoplasm   The effect produced is that often seen in the nucleus of the fully developed club-shaped forms to be next described

Nucleus —The nucleus is a single sharply-defined and compact body surrounded by the halo previously described   It is too small for any details of internal structure to be readily made out

Division —So far as has been ascertained no division takes place at this stage of development

Effect on cell —The effect on the containing cell described in connection with the pseudocysts holds here but in an exaggerated degree

Cytology of the forms of B canis *seen in the nymph of* R sanguineus
*eleven to fifteen days after it has completed its meal and
dropped off the infected dog*

About this stage in the life of the tick two stages in the cycle of development of *B canis* may be seen These are (*a*) The culmination of the development of the pseudocysts resulting in the formation of club-shaped bodies (*b*) The migration of the club-shaped bodies to the muscles and muscle-sheaths and their development there into secondary sporoblasts

(*a*) *Club-shaped bodies in the pseudocysts* (Plate XXXIV, fig 13)

Shape —The name given to these bodies indicates roughly their shape They have a generally fish-shaped body, one extremity being more bluntly pointed than the other Some of the bodies show a slight constriction in their middle which is an indication of their method of development as described by Christophers (1907) These are the forms as first developed from the pre-existing primary sporoblasts Their greatest breadth bears a proportion of 1 5 to their length At a later stage of development the club-shaped bodies are more elongated There is often an indistinct cup-shaped organ at the extremity of the broad end of the club

Size —The length of the club-shaped bodies in the pseudocysts varies The average length and breadth, as measured from the bodies in one pseudocyst, was $8 9\mu$ by 2μ but the measurement is greatly affected by optical foreshortening in many cases where the bodies are lying in many differently inclined planes and much longer free forms may frequently be seen

Cytoplasm —The cytoplasm has the same ground-glass appearance described for preceding forms There are no cytoplasmic inclusions but in some cases vacuole-like rarefactions may be seen in the cytoplasm Around the nucleus, which is situated about the junction of the anterior third with the posterior two-thirds of the parasite, is a very distinct rarefaction of the cytoplasm forming a well-marked halo round the chromatin In many cases there appears to be a distinct membrane bounding the halo in which case the whole structure—halo containing the chromatin dot—would appear to constitute the nucleus The cytoplasm is often denser in appearance from a point half-way between the nucleus and the broad end of the club up to the narrow end

One other structure often to be seen is a linear rarefaction of cytoplasm forming a pale transverse band across the parasite behind the area occupied by the nucleus This may be a remnant of the achromatic lines seen in the pre-club forms

Nucleus —The nucleus is a well-marked body consisting of a central compact more or less spherical mass of chromatin in which no further structure can be made out and surrounded by a pale area which appears to be itself bounded peripherally by a nuclear membrane This appearance is more or less constant in fully developed club-shaped bodies and we presume the whole structure to represent the nucleus, of which the chromatin is concentrated in a central mass

Division—So far as has been observed no division of club-shaped bodies, as such, occurs

Effect on cell—The club-shaped bodies in the pseudocysts represent the culmination of development of the latter and the containing cell is a mere envelope for the pseudocyst It is often impossible to distinguish either cytoplasm or nucleus of the containing cell

(b) Secondary sporoblasts

The club-shaped bodies in the mature pseudocyst rupture the latter and migrate to the muscles where they enter the muscle substance, lying between the fibres, or invade the cells of the muscle-sheath In either situation they round up, or at least lose their typical club-shaped form, and proceed to divide The results of their division are the secondary sporoblasts

Shape—The typical shape of the secondary sporoblast is ovoid and, when contained in the cells of the muscle-sheaths it tends to retain that shape When, however, it is situated in the substance of the muscle the pressure of the surrounding muscle-fibres tends to make it somewhat elongate and, when several are arranged in a line, they may become even cubical in shape due to squaring of their contiguous ends (Plate XXXV, fig 21) When the process of division is complete the final shape of the secondary sporoblasts is an ovoid or slightly elongate form (Plate XXXIV, figs 17 and 18)

Size—The secondary sporoblasts vary very greatly in size, tending to be larger in their earlier stages when they first reach the muscle and commence to divide and becoming smaller as the result of continued division All variations in size may be found between extremes of $0 8\mu$ and $3 8\mu$

Cytoplasm—The cytoplasm does not differ essentially from that of the primary sporoblasts and the description given in connection with the latter will suffice

Nucleus—The nucleus is a single compact mass of chromatin with no distinct halo round it as in the case of its predecessor in the club-shaped body It is usual, however, until division is complete, to find many, if not the majority, of the secondary sporoblasts displaying two or more aggregations of chromatin, the result of rapid division No internal structure in the nucleus can be made out

Division—At the stage we are considering division is very active and single, binucleate and quadrinucleate, forms may be seen These multinucleate forms almost give one the impression that a process of schizogony is in progress but this is probably incorrect The method of division is apparently a simple binary fission The nucleus elongates and divides into two It should, however, be mentioned that occasional pictures are seen closely resembling mitosis and a few such forms are illustrated in Plate XXXV, fig 20 Whatever the process may be, the division of the cytoplasm lags behind that of the nuclei with the production of the multinucleate parasites resembling schizogony forms Eventually the cytoplasm condenses round new nuclei to form daughter secondary sporoblasts which appear to grow and themselves repeat the process of division When the process of division is completed the final secondary sporoblasts are comparatively small ovoid or elongate bodies measuring about $1 2\mu$ in their longest diameter (Plate XXXIV, figs 17 and 18)

Effect on cell—The parasites occupying cells of the muscle-sheaths bulge out the latter in such a way as to form protuberances from the general regular contour

of the muscle   The parasitized cells, however, do not seem to be physiologically adversely affected as it is seen that the nuclei stain perfectly

The parasites lying within the muscular structure (Plate XXXIV, fig 15, and Plate XXXV, figs 21 and 22) do not appear actually to be lying in cells but rather in a space formed by the separation of the muscular fibres due to their presence Whether this is actually so or whether there are intramuscular prolongations of the external sheath around muscular fasiculi is not certain   If such prolongations exist it is possible that it is in the cells of these prolongations that the parasites lie

Cytology of the forms of B canis *seen in the newly-hatched adult twenty to twenty-one days after the nymph of* R sanguineus *has completed its meal and dropped off the infected dog*

The forms seen at this stage are identical with those described in the last section, i e , peri- or intramuscular forms, and need no further mention

Cytology of the forms of B canis *seen in the adult* R sanguineus, *developed from an infected nymph, after the adult has commenced its blood meal on a dog*

This resolves itself into a description of the forms of *B canis* found in the salivary glands of the adult   As described in a previous section the parasites are only found in the glands after the commencement of feeding   They may be present in two situations —

(*a*) In the salivary acini proper

(*b*) In the poison acini

(*a*) *In the salivary acini proper* —The parasites in the salivary glands, being the results of division of the secondary sporoblasts, may be termed sporozoites and are the form leading to the infection of a new host when the tick feeds

Shape —The typical shape of the sporozoites in the salivary acini is globular or ovoid   This is best seen at the periphery of a group, the outer parasites not being subject to the distorting pressure exercised on those more centrally placed The shape of the parasites found in the salivary ducts, and therefore presumably the actual and final infective form, is somewhat less regular and it often takes the form of a small oval body with a centrally placed nucleus   More or less elongated forms or other irregularities in shape may also be seen

Size —The size of the sporozoites is comparatively uniform, not only in the same acinus but throughout the infected gland   The average diameter is about 1 9μ The form finally produced by repeated divisions may be somewhat smaller as may some of the forms seen in the salivary ducts

Cytoplasm —The cytoplasm has the same ground-glass appearance previously described for the primary sporoblasts and shows a similar absence of inclusions or vacuoles

Nucleus —The nucleus of the mature sporozoites is a small compact mass of chromatin centrally placed.   This mass is too small to allow of the differentiation

of any internal structure but careful focusing will sometimes reveal a slight rarefaction in the cytoplasm surrounding the chromatin mass   It is possible, therefore, as in the case of the club-shaped bodies and their immediate precursors, that this apparent rarefaction also forms part of the nuclear body, the central mass constituting the entire chromatin content

The nuclei of the parasites in which the process of continued division is not yet complete are much more bulky, at least as regards the chromatin mass, and the latter is also more irregular in shape in accordance with its state of activity at the time of fixation

Division—Division in the sporozoites is by a process of simple binary fission The compact nucleus elongates and divides into two   There is often a lag in the division of the cytoplasm but this is not so marked a feature as in the case of division of the secondary sporoblasts   The result of repeated division of the sporozoites is to produce finally a slightly smaller globular or possibly oval or somewhat elongate end form with a single small centrally placed mass of chromatin which is the infective form

Effect on cell—The invasion of the cells of a salivary acinus by the parasites produces very little apparent deleterious effect on the secreting cells in the earlier stages of the invasion   This is evidenced by the normal appearance of the nuclei and cytoplasm of the cells   In the later stages, when the parasites by continued multiplication have almost entirely replaced the cytoplasm of the secreting cells, what remains of the latter becomes vacuolated and functionless   The nuclei of the secreting cells at this stage are also often completely disintegrated and the chromatin instead of being in small sharply-defined discrete masses takes on a diffuse though deep stain giving the appearance of a dense amorphous mass of chromatin   Such acini eventually become mere containing bags for immense masses of sporozoites and entirely functionless as regards production of the salivary secretion   The same acinus often shows normal fully functioning cells as well as cells completely occupied by parasites

(b) *In the poison acini* (Plate XXXVI, fig 26)

The description of the forms found in the salivary acini as well as of the effects produced by them on the containing cells may be applied to the forms found in the poison acini with certain modifications which will be mentioned   The forms seen in the poison acini tend to have a much larger eccentrically placed chromatin mass   This is probably due to the fact that the infections of poison acini seen were in a comparatively early stage of the invasion while multiplication of the parasites was still very active   The illustration (Plate XXXVI, fig 26) shows how the active division of parasites results in groups resembling morulas The coalition of these finally leads to a close aggregation of the parasites which may eventually practically fill the entire poison acinus   The final stage reached in the salivary acini, viz, of small globular or ovoid sporozoites each with a single small nucleus has not been seen in a poison acinus but this may have been due to the earlier stage of infection in the specimens examined   The cytoplasm of the poison acinus forms stains darkly with iron hæmatoxylin but, on differentiation, tends to show more or less vacuolation, a characteristic absent in the forms in the ordinary salivary acini,

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Cytology of the forms of B canis *found in the ovaries of* R sanguineus *or in the ova themselves*

(a) *Club-shaped forms* (Plate XXXVII, figs 29 and 30)

Shape —This has already been dealt with in the section describing the culmination of development in the pseudocysts the only difference being that the club-shaped bodies in the ova or ovaries are possibly longer on an average

Size —This varies possibly more with the state of contraction of these motile bodies than from any other cause Forms as long as 13μ when measured in a straight line have been encountered, the proportion of length to breadth in these forms being 13 19

Cytoplasm —This does not differ from the description of that in club-shaped forms previously dealt with The halo round the nucleus and the transverse rarefaction of cytoplasm behind the nucleus are often marked features

Nucleus —The chromatin of the nucleus may be of the compact spherical form described in a previous Section dealing with club-shaped forms or it may be elongate or otherwise irregular, indicating the commencement of the series of divisions leading to the forms to be next described

Division —Division of the nucleus seems to be initiated by a protrusion from the side of the spherical mass of chromatin This protrusion, as it develops, leads to the general lengthening out of the chromatin into a band in a direction transverse or slightly diagonal to the long axis of the club-shaped body This is accompanied by a shortening and rounding of the body which becomes bulky, losing its typical club-shaped form Division of the cytoplasm may now result or the chromatin may divide into two and the two daughter nuclei again initiate division while the cytoplasm lags in its response

Effect on cell —The presence of the club-shaped bodies in the ova has no effect on the latter apart from their mere mechanical presence as foreign bodies

(b) *Forms of irregular shape but tending to be ovoid when units*

Shape —When the parasites are seen as units the shape is usually an ovoid (Plate XXXVII, figs 35 and 36) The outline in such forms is regular When division is in progress the shape of the parasites varies within wide limits and may be ovoid, elongate, angular or show other irregularities in outline (Plate XXXVII, figs 31, 32, 33 and 34)

Size —The size varies greatly but, here again, the variations are largely dependent on phases of division and the resting units are more uniform in dimensions The latter forms vary between 1 4μ and 2μ in their longest diameters

Some of the largest forms seen were in empty and shrunken egg follicles from which the ova had been discharged Such forms might measure as much as 5 6μ in their longest diameter (Plate XXXVII, fig 32)

Cytoplasm —The cytoplasm was ground-glass like and usually uniform in the smaller forms Some of the larger forms occasionally showed signs of commencing vacuolation of the cytoplasm and also rarefaction around the nuclei producing a faint halo effect

PLATE XXXI

1

2

Fig 1   Epidermal layer of cells in nymph of *R sanguineus*
 ,,   2   do   high power appearance.

PLATE XXXII.
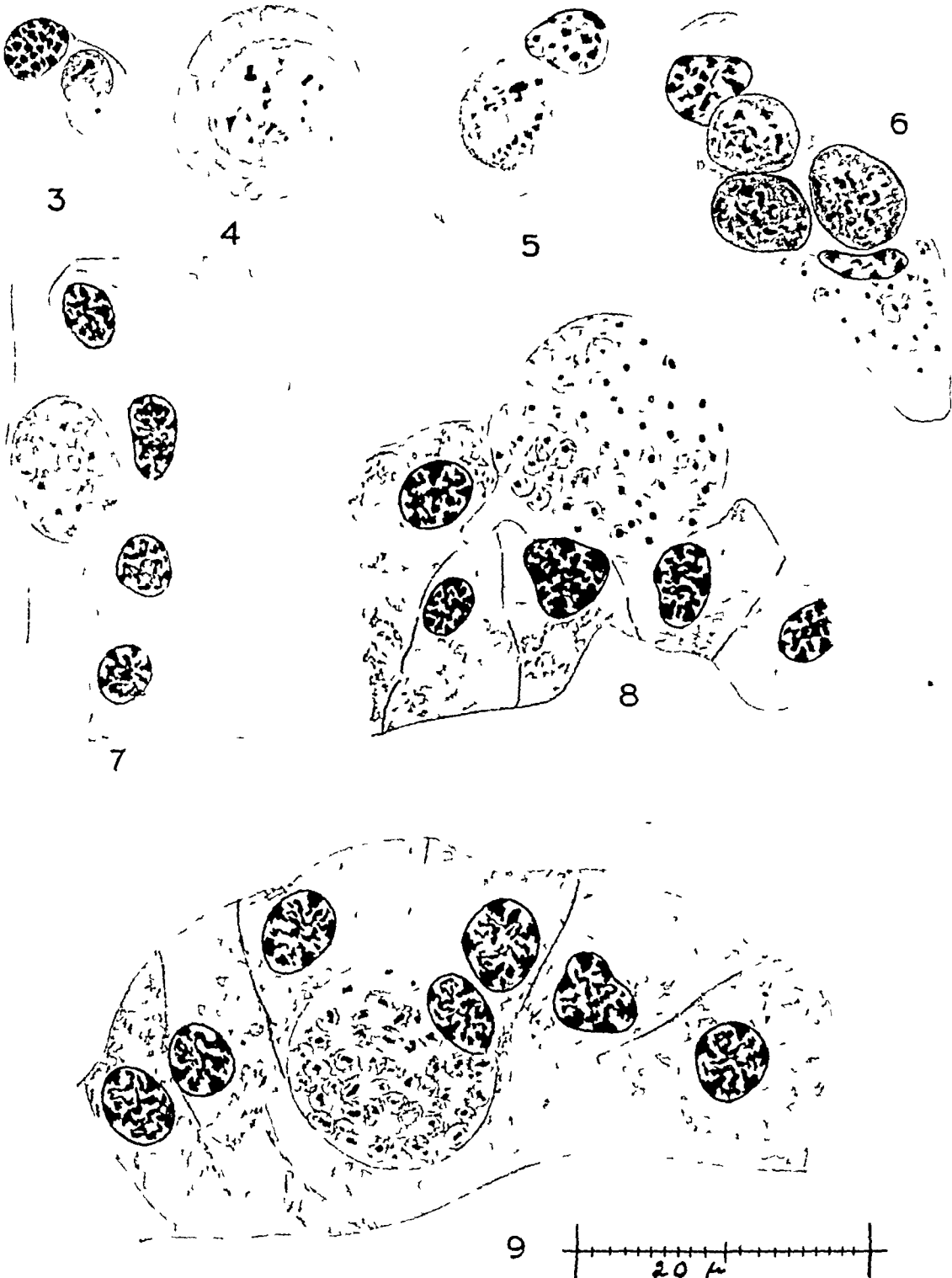
Figs 3, 4, 5, 6 and 7   Early developmental stages leading up to the formation of pseudocysts
„   8 and 9   Developed pseudocysts under the epidermal layer

PLATE XXXIII.

10

11

12

20 μ
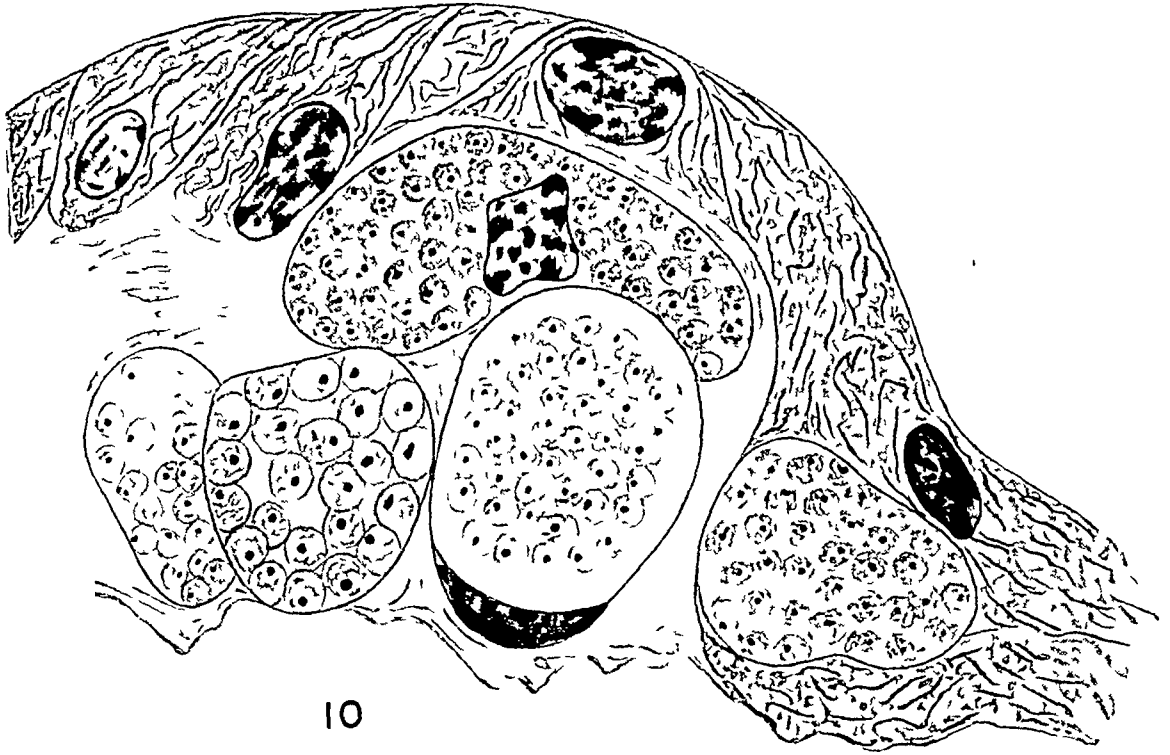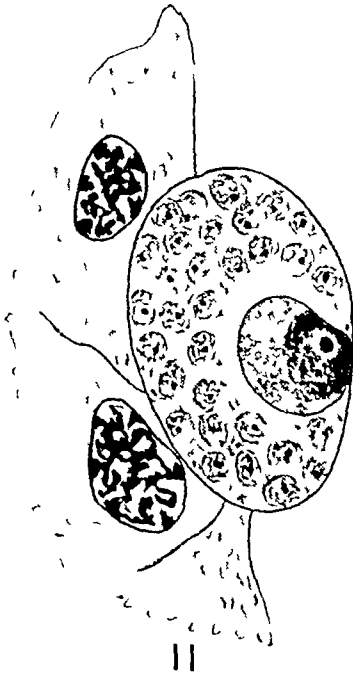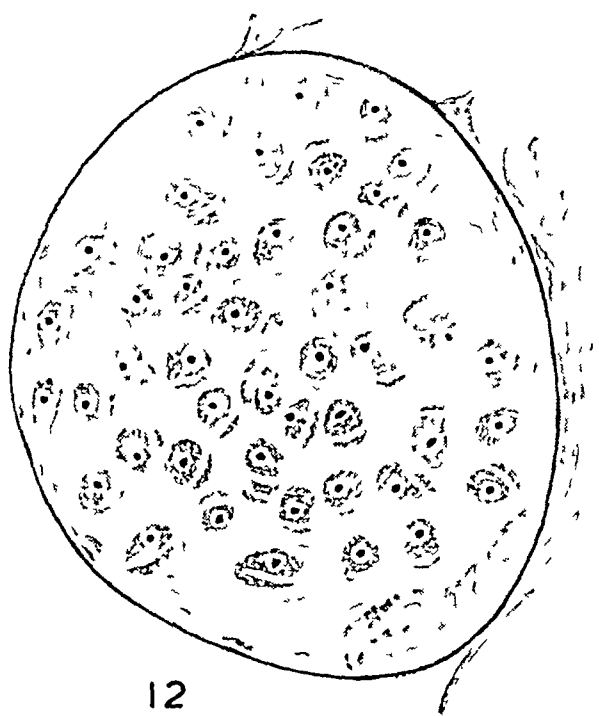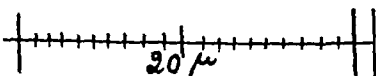
Fig. 10   Same as Plate XXXII, figs 8 and 9

„   11   Pseudocyst showing an early developmental form in the same cell

„   12   Pseudocyst containing pre club forms   Note the achromatic lines

PLATE XXXIV
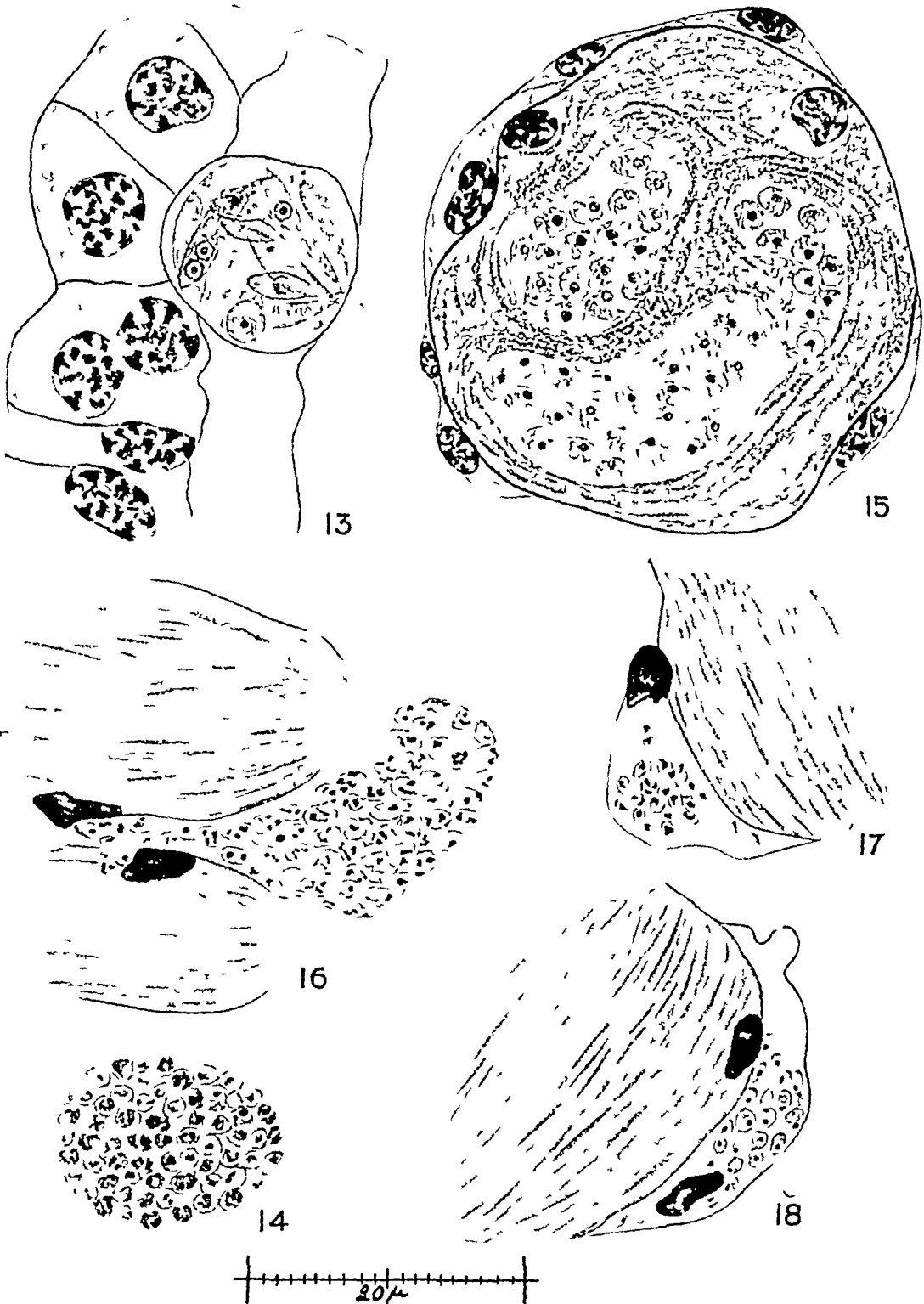
Fig 13 Fully developed pseudocyst containing clubs
Figs 14, 15, 16, 17 and 18 Secondary sporoblasts in the muscles or muscle sheaths of nymphal ticks

PLATE XXXV

20 μ
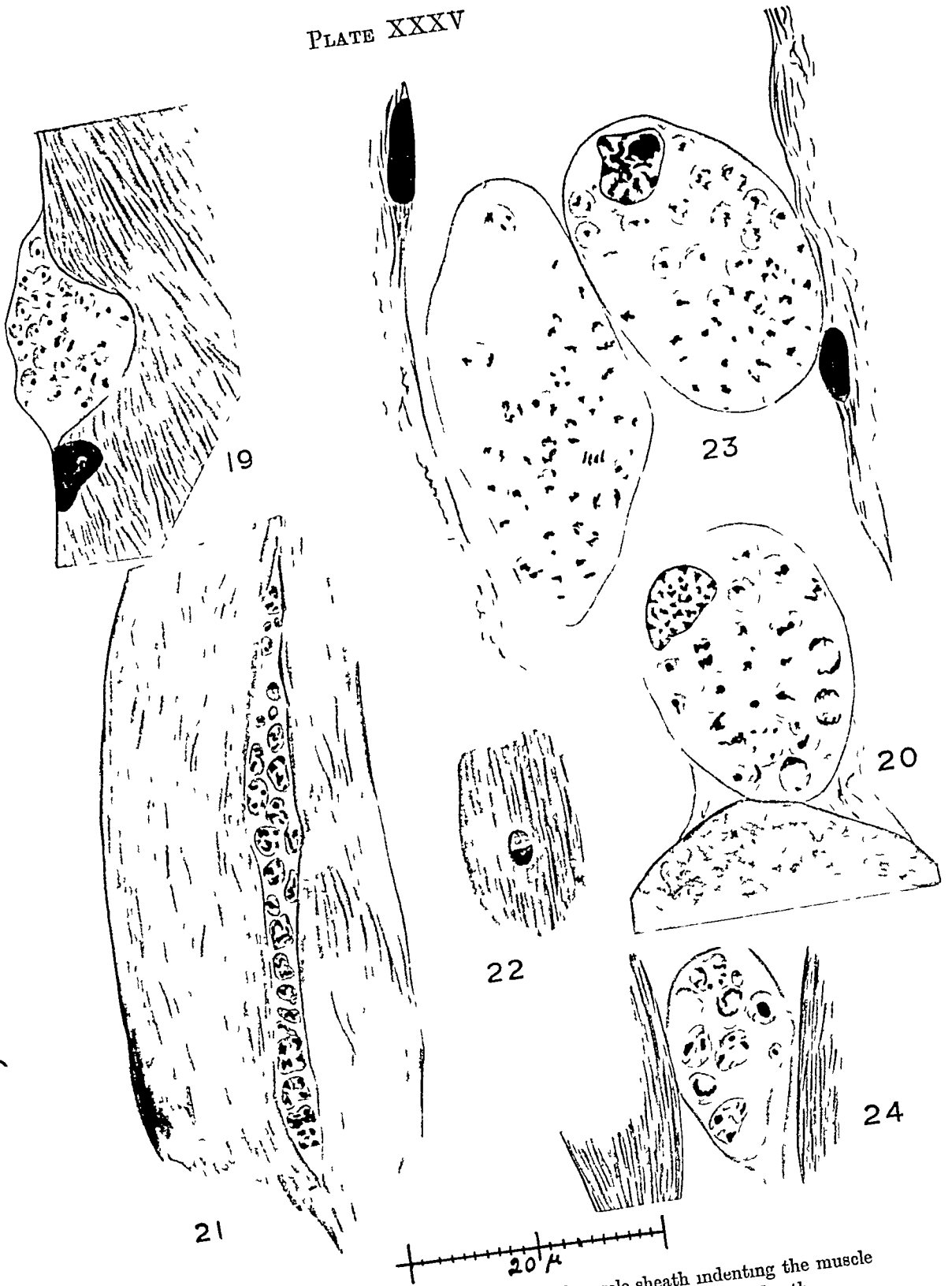
Fig 19   Secondary sporoblasts in cell of muscle sheath indenting the muscle
  „   20   Secondary sporoblasts in pedunculated cell of muscle sheath
  „   21   Secondary sporoblasts in linear arrangement between muscle fibres
  „   22   Single dividing secondary sporoblast in muscle substance
Figs 23 and 24   Secondary sporoblasts in muscle showing stages in multiplication
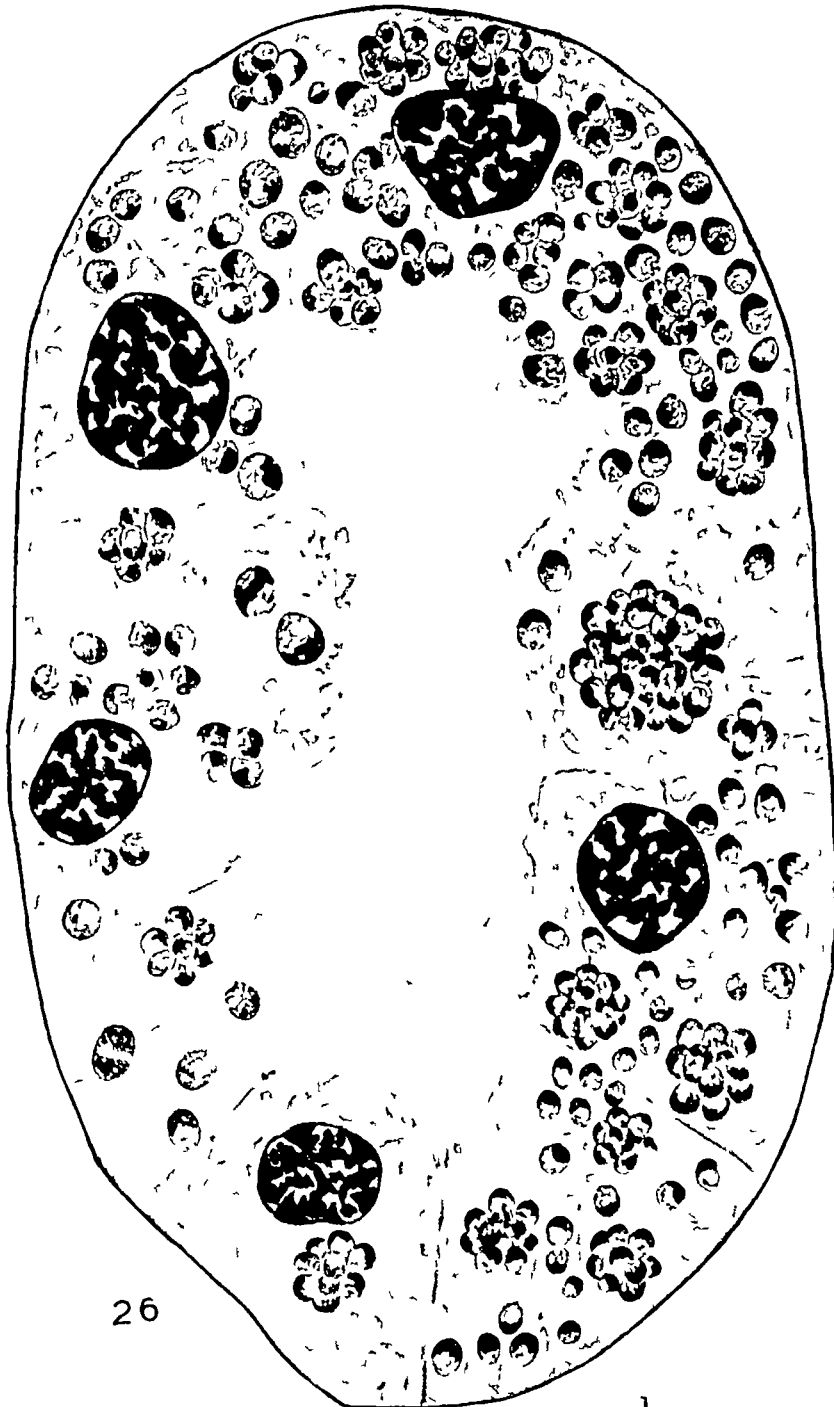
PLATE XXXVI

26
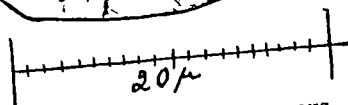
Fig. 26. Infected poison acinus

PLATE XXXVII

Figs 29 and 30   Club shaped bodies from infected ova

,,   31, 32, 33, 34, 35, 36, 37, 38 and 39   Various forms of *B canis* as found in infected
ova or ovarian follicles

,,   40 and 41   Forms of *B canis* found in larvæ hatched from infected ova

PLATE XXXVIII.
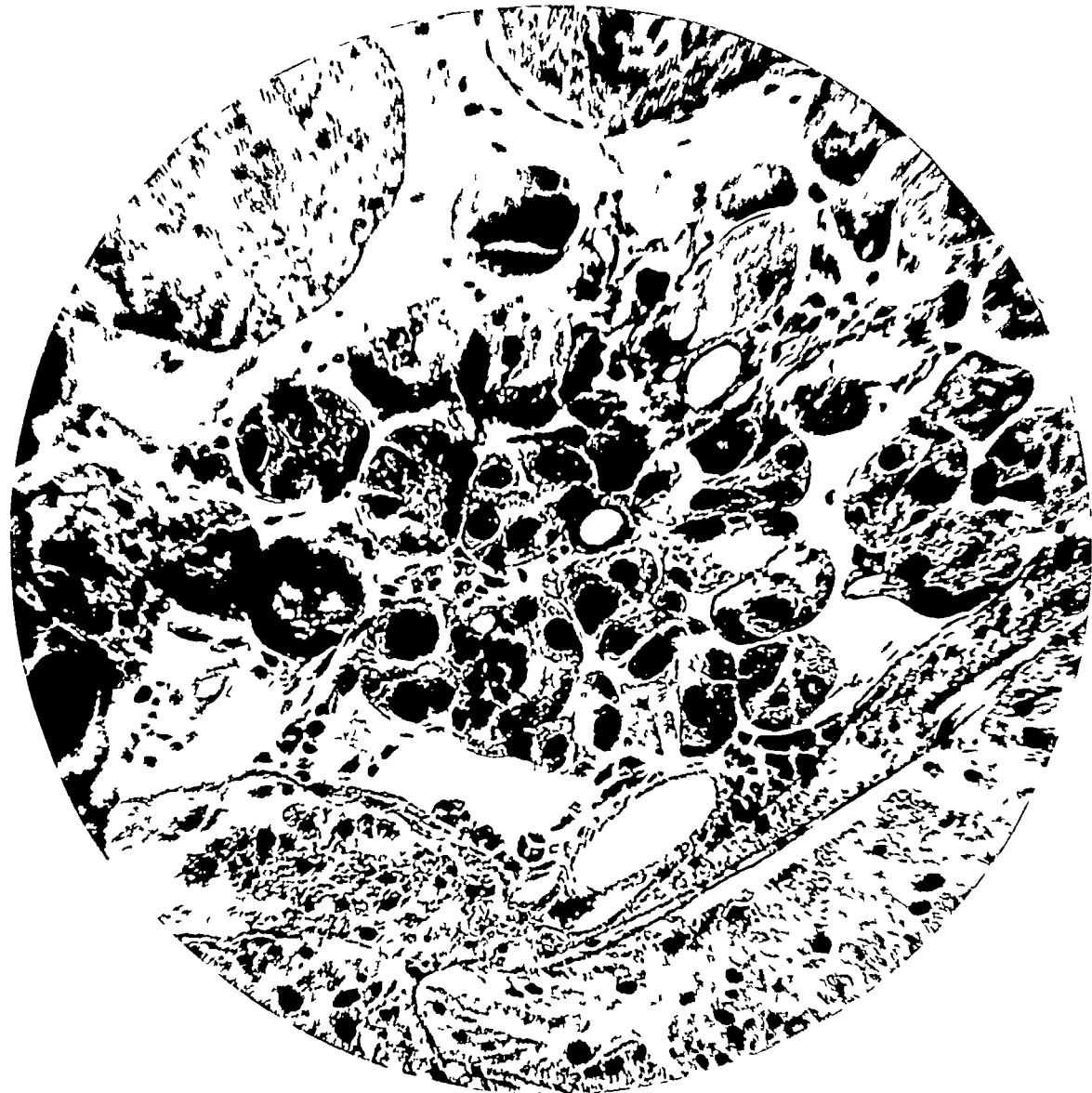
43

Fig 43   Group of infected salivary acini   The dark areas are nuclei of the acinar cells   Practically every acinus shown is heavily infected

PLATE XXXIX

42

27

(a)

(b)

(c)
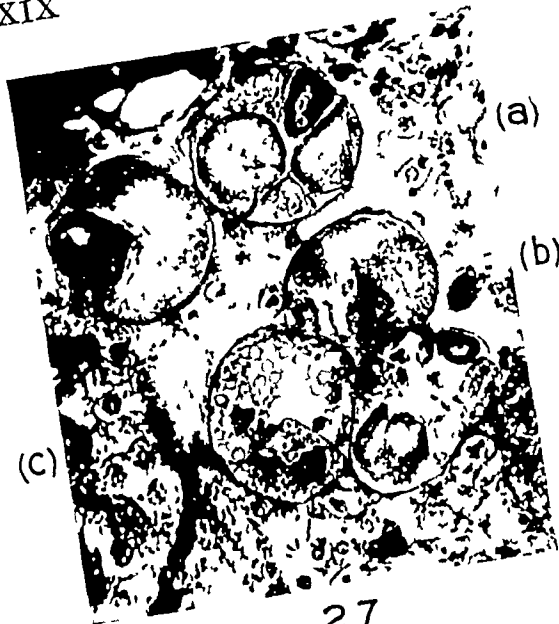
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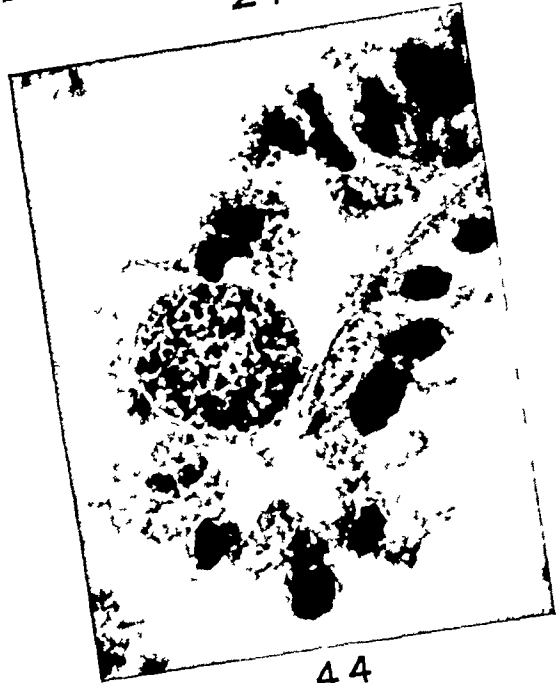
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25

Fig 12   An infected salivary acinus under a high power
"   25   A line of salivary acini extending along one side of a tick
"   27   (a) Acini completely filled with parasites
        (b) Acinus with one cell infected
        (c) Acinus with one cell infected but also containing zymogen granules in the other cells   One or two infected acini are
"   28   Exhausted acini which have got rid of their secretion
        also seen
"   44   A developed pseudocyst under the epidermal layer

Nucleus—The nucleus of the small units is a compact rounded mass of chromatin too small to show any internal structure   That of dividing forms is often very bulky (Plate XXXVII, figs 32, 33 and 34)   Around these forms appears to be a distinct nuclear membrane and the chromatin is aggregated into coarse granules on a somewhat more lightly stained background

Division—Division appears to be by simple binary fission   The nucleus elongates and divides into two daughter nuclei which pass to opposite poles of the parasite   The cytoplasm may now divide and form two complete daughter parasites or the daughter nuclei may themselves divide before division of the cytoplasm is complete   In this manner comparatively large apparently multinucleate parasites may be formed but usually the division of the cytoplasm is at least indicated in such forms   These formations ultimately result in smaller or larger groups of more or less adherent small forms—the unit forms already described

Effect on cell—The forms under consideration produce no appreciable effect on the ova or egg follicles within which they are contained and the ova are in no way prevented from completing their development—hence the capacity for hereditary infection

Cytology of the forms of B canis *seen in unfed larvæ of* R sanguineus *hatched from infected eggs* (Plate XXXVII, figs 40 and 41)

Shape—The shape of the parasites is an ovoid with regular contour

Size—The size is fairly uniform in the same larva   The average measurement of the longest diameter was $1\ 7\mu$ but variations between $2\ 4\mu$ and $0\ 6\mu$ are to be found

Cytoplasm—The cytoplasm has the ground-glass appearance previously described and is devoid of vacuoles or inclusions

Nucleus—The nucleus appears to be always a single compact mass of chromatin situated more or less centrally in the parasite   No internal structure can be made out

Division—No dividing forms were seen

Effect on cell—As already stated the parasites at this stage appear to be extracellular—situated in the undifferentiated yolk which is still present in the larvæ

SUMMARY

1   A description is first given of the forms of *B canis* seen at different stages of its life history in the dog-tick, *R sanguineus*

2   A consecutive description of the life-history is then outlined from the time the parasite is ingested by the tick up to its transmission during the act of feeding of the latter to a fresh host

3   The morphology of the different stages is described

For constant and highly skilled technical assistance he is immensely indebted to his assistant Mr C S Swaminath whose unrivalled knowledge of the type of work involved rendered possible the examination of an immense amount of material Without his help the author would not have had the time to undertake the investigation

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STUDIES ON TYPHUS IN THE SIMLA HILLS

Part IV.

THE RÔLE OF THE RAT FLEA IN THE TRANSMISSION OF TYPHUS

BY

LIEUT.-COLONEL G COVELL, M D, D P H, I M S,

AND

D R MEHTA, M SC, PH D

(*Inquiry under the Indian Research Fund Association*)

A STRAIN OF TYPHUS RECOVERED FROM NATURALLY INFECTED RAT FLEAS

ON 2nd September, 1935, an emulsion in normal saline of 121 *Xenopsylla cheopis* and 2 *Ceratophyllus* sp, collected from 15 wild rats (*Rattus rattus* group) trapped at Sabathu, was injected intraperitoneally into a guinea-pig This animal developed a typical scrotal reaction and fever on the 7th day after inoculation The strain (FL) has since been maintained in guinea-pigs by subpassaging with emulsions of spleen intraperitoneally, and is now in its 17th generation Out of 50 guinea-pigs used as passage animals, 38 have developed typical scrotal reaction and pyrexia, 3 scrotal reaction without pyrexia, and 7 have reacted with fever only Typical intracellular *Rickettsiæ* have been repeatedly observed in scrapings from the tunica vaginalis of guinea-pigs and white rats inoculated with passage virus The Weil-Felix test has been carried out against alcoholized suspensions of *B proteus* OX19, OXK and OX2 on a number of white rats The sera of these animals has in the majority of cases given a positive result with OX19, the highest titres recorded being 1 1,000 (on two occasions, once on the 10th day and once on the 15th day after inoculation) No positive results have been obtained with OXK or OX2 The reaction of guinea-pigs and white rats to infection with this strain appears to be the same as in the case of strain R, previously recovered from the brains of wild rats caught in the same area, and described in detail in a previous paper (Covell, 1936*b*) Guinea-pigs infected with the flea strain have been shown to be immune to

subsequent infection with the rat strain, and vice versa, except on one occasion, when a guinea-pig which had recovered from an infection with strain R developed a typical scrotal and febrile reaction when infected with strain FL six weeks later

Experimental transmission of a typhus strain by the rat flea, *Xenopsylla cheopis*

On 3rd September, 1935, a white rat WR/1 was inoculated intraperitoneally with passage virus (guinea-pig spleen emulsion) from a typhus strain originally derived from the brains of wild rats (strain R)

On 12th September, 46 rat fleas (*Xenopsylla cheopis*), collected from 12 wild rats trapped at Kasauli and Sabathu, were placed on WR/1, which was kept in a glass jar, the mouth of the jar being covered with muslin stretched over wire netting   WR/1 was found to be in a dying condition on 16th September, and was killed   The Weil-Felix reaction was positive with OX19 in a dilution of 1   350

Thirty-four fleas collected from WR/1 were placed on a second white rat WR/2 on 16th September, and were allowed to remain on it for 3 days, after which it was chloroformed, cleared of ectoparasites and placed in a fresh clean jar This rat was killed on 30th September   The Weil-Felix test was negative Emulsions of the spleen and brain of the rat were injected intraperitoneally into 4 guinea-pigs   None of these developed any scrotal reaction, but one showed a very slight rise of temperature (102 2°F and 102 4°F) on the 8th and 9th days, and subpassage was made into 2 other guinea-pigs and 2 white rats   Neither of the guinea-pigs reacted, and both were subsequently proved to be non-immune to infection with the parent strain   The Weil-Felix test proved negative with the sera of the 2 white rats

Nineteen fleas were recovered from WR/2 on 19th September   These were placed on a third white rat WR/3, and were allowed to remain on it till 28th September, when it was chloroformed and cleared of ectoparasites   The animal was killed on 8th October, and an emulsion of its brain was injected intraperitoneally into 2 guinea-pigs   Both of these developed typical scrotal reaction and fever, commencing on the 9th and 12th days respectively   The strain was maintained in guinea-pigs through 9 generations to enable cross immunity experiments to be carried out, and was then dropped   The incubation period shortened to 5—7 days after the first subpassage, and the reaction produced in guinea-pigs and white rats appeared to be identical with that produced by the parent strain   Cross immunity was shown to exist between the substrain and the parent strain R, and also with the strain FL referred to above   Typical *Rickettsiæ* have been observed in scrapings from the tunica vaginalis of passage guinea-pigs and white rats   The Weil-Felix reaction has been positive in white rats with *B proteus* OX19 up to a titre of 1   500, but negative with OXK and OX2

On 8th October, a fourth white rat WR/4 was placed in the jar in which WR/3 had been kept, after having been chloroformed and completely cleared of ectoparasites[*]   Three fleas (*X cheopis*) were caught on this rat on 21st October

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[*] This procedure was carried out as a preliminary routine with each rat used in these transmission experiments

They were killed, and a smear was made from the midgut in each case and stained with Giemsa Rickettsiæ, morphologically indistinguishable from those previously seen in scrapings from the tunica vaginalis of passage guinea-pigs and white rats, were present in enormous numbers in one of the smears

On 23rd October, 25 specimens of *X cheopis*, collected from wild rats, were placed on WR/4, which had first been chloroformed and cleared of ectoparasites On 26th October, 3 fleas were removed and killed, and a smear was made from the midgut of each Rickettsiæ in scanty numbers were observed in one of these smears, whilst the other two were negative On 28th October, 3 more fleas were removed and examined Rickettsiæ in large numbers were observed in all three of the smears

The results of the above experiments may be summarized as follows —

(1) A batch of 34 rat fleas (*X cheopis*) which had been allowed to feed during a period of 3 days on a white rat experimentally infected with a strain of typhus originally derived from the brains of wild rats, and then placed on a normal white rat for 3 days, failed to transmit the virus

(2) The virus was successfully transmitted by survivors from the same batch, which had been removed from the second white rat after 3 days, and allowed to remain on a third white rat for 9 days

(3) The clinical and serological reactions produced in guinea-pigs and white rats after passage through the rat flea were identical with those produced by the parent strain The substrain exhibited cross immunity with the parent strain, and also with a strain originally derived from fleas caught on wild rats

(4) Rickettsiæ were observed in scanty numbers in a smear from the midgut of one out of 3 fleas which had been killed after having been allowed to feed on an infected white rat during a period of 3 days, smears from the other two fleas being negative

(5) Rickettsiæ in large numbers were observed in smears from the midgut of 3 fleas which were killed after having been allowed to feed on an infected white rat during a period of 5 days

(6) Rickettsiæ in enormous numbers were observed in a smear from the midgut of a flea 35 days after it had been removed from an experimentally infected white rat on which it had been allowed to feed during a period of 3 days

No Rickettsiæ were observed in a series of smears from the midgut of normal laboratory bred fleas *

DISCUSSION

During the last few years evidence has accumulated regarding the rôle played by fleas in the transmission of typhus strains in many countries Dyer and his colleagues (1931*a*, 1931*b*, 1931*c*, 1931*d*, 1931*e*, 1931*f*, 1932*a*, 1932*b*) in the United States have shown that the virus of endemic typhus is present in rat fleas collected from wild rats under natural conditions , that the disease is readily transmitted by

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* As has been pointed out by Mooser and Castaneda (*loc cit*), Rickettsia like organisms are some-times seen in preparations made from normal fleas, but they are usually larger than true Rickettsiæ, and are found in smaller numbers Moreover, they do not take on the peculiar coloration with Giemsa stain which is characteristic of Rickettsiæ, and they are never intra cellular

fleas from rat to rat experimentally, that the virus is present in the fæces of infected fleas, and may be transmitted by rubbing macerated infected fleas or the fæces of infected fleas into the abraided skin of guinea-pigs, that infected fleas may retain the infection for as long as 52 days, and that the virus multiplies enormously in *X cheopis* Despite repeated attempts, they were unable to transmit typhus by the bite of infected fleas when fæces were not allowed to come into contact with the skin of the experimental animals used, nor did they obtain any evidence to indicate that the virus may be transmitted to the offspring of infected fleas through the egg

Mooser and Castaneda (1932) transmitted the virus of Mexican typhus by means of *Xenopsylla cheopis, Leptopsylla musculi, Ceratophyllus fasciatus, Cteno-cephalus canis* and *Ctenocephalus felis*, and showed that the virus multiplied abundantly in all these species They were also able to infect *Pulex irritans* They failed to find *Rickettsiæ* in normal fleas, but observed them in large numbers a few days after the fleas had fed on infected rats They demonstrated the presence of *Rickettsiæ* in the epithelial cells of the stomach and in the epithelial lining of the Malpighian tubules None were seen in the salivary glands and ducts They noted that *Rickettsiæ* were scarce in the lumen of the fleas' intestines and explained this fact by the presence of a peritrophic membrane* covering the mucosa of the entire stomach They considered that the *Rickettsiæ* entered the gut almost exclusively by the route of the Malpighian tubules They explained the relative harmlessness of the flea in the transmission of typhus to man by the absence of infection in the salivary apparatus, the presence of the peritrophic membrane, the fact that flea bites are less irritating to most human beings than those of lice (leading to less scratching), and that the body of most fleas is more strongly chitinized than that of lice (hence the danger of crushing a flea against the skin is less than with lice) They also stressed the disinclination of the rat fleas *Xenopsylla* and *Ceratophyllus* for man, and the fact that since typhus does not kill rats under natural conditions, there is little occasion for the fleas to leave their normal host

The fact that fleas can carry the virus of typhus strains has also been demonstrated in the case of ship typhus of the French seaports, in the endemic typhus of Greece and other countries in the Mediterranean area and in Manchuria, whilst the sporadic type of typhus occurring in South Africa is also suspected to be carried by rat fleas

Nicolle and his colleagues (1933a, 1933b) were unable to infect rats by the bites of fleas, but were able to transmit the disease by feeding rats on an emulsion of infected fleas They considered that rats are infected in nature either by eating infected fleas, or by eating the bodies of other rats which are already infected

Lewthwaite (1935) has shown that *X cheopis* under experimental conditions can transmit the virus of the urban typhus of Malaya from rat to rat On the other hand, in a series of experiments with ticks (*Dermacentor andersoni* and *Rhipicephalus sanguineus*) he obtained no evidence that these insects could transmit or even acquire the viruses of either the rural or the urban form, a finding of great interest to workers in India

The observations made during the present inquiry have proved that the wild rat is a reservoir of typhus in the Simla Hills area, that the virus is present in rat

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* Wigglesworth, however, states that this structure is not present in fleas ('Insect Physiology, 1934, p 48)

fleas under natural conditions, and that the virus can be transmitted experimentally by means of fleas from rat to rat   The experiments detailed above also indicate that the virus multiplies abundantly in the rat flea, as has been demonstrated by workers in other countries

As has been noted in a previous paper (Covell, 1936a), a crop of typhus fever cases (XK type) has been observed to occur in each of the last four years in the Simla Hills a few weeks after the end of the rainy season   It is at this time of the year that fleas are especially prevalent   In 1935 the rainy season began and ended

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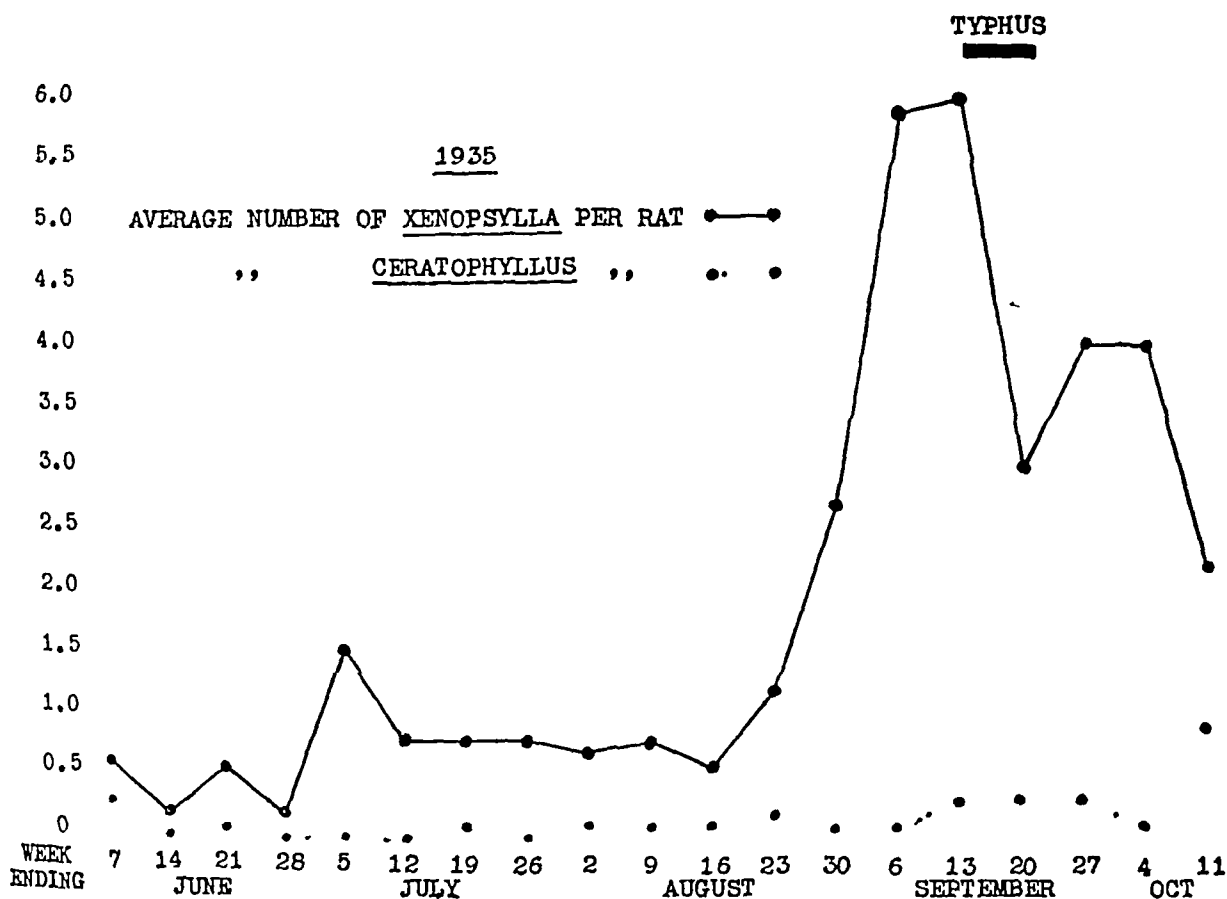
TYPHUS

some three weeks later than usual , the increased prevalence of fleas was also noted to be later than usual , and the few definitely proved cases of typhus which were observed (7 in number) all had their onset between the 13th and 21st of September, whereas in 1933 and 1934 the first cases were observed during the last few days of August   During the present investigation, a survey of the ectoparasites of rodents is being carried out, which will form the subject of a later paper   The data as regards the incidence of fleas on wild rats from June to mid-October are given in the accompanying Chart, which brings out in a graphic manner the relationship

between the peak of maximum prevalence of *X cheopis* and the September outbreak of typhus cases   There is no evidence to show that the disease is prevalent among outdoor workers, and it is perhaps significant that the three Indian cases observed in September 1935 and one of the four British cases were all cooks

The available evidence points to the rat flea as being the most probable insect vector of typhus in the Simla Hills area, but more experimental work is necessary before the possibility that other ectoparasites may be concerned is dismissed

ACKNOWLEDGMENTS

SUMMARY

(1) A strain of typhus has been recovered from rat fleas collected from wild rats, and has so far been maintained in guinea-pigs for 17 generations

(2) A strain of typhus originally derived from the brains of wild rats has been transmitted from an experimentally infected white rat to a normal white rat by means of the rat flea, *Xenopsylla cheopis*   The clinical and serological reactions produced in laboratory animals by infection with the strain were unaltered by its passage through the flea

(3) *Rickettsiæ* morphologically indistinguishable from those observed in laboratory animals inoculated with passage virus have been demonstrated in fleas fed on experimentally infected white rats   Our results indicate that there is a great multiplication of *Rickettsiæ* in *Xenopsylla cheopis*, thus confirming the observations of workers in other countries

(4) Epidemiological evidence points to the rat flea as a probable natural vector of typhus from rat to rat and from rat to man in the Simla Hills area

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THE GROWTH OF EMBRYONIC NERVOUS TISSUE IN PLASMA TAKEN FROM VITAMIN A DEFICIENT FOWLS AND RATS.

BY

W R AYKROYD

AND

G SANKARAN

(*From the Nutrition Research Laboratories, I R F A , Coonoor*)

In some previous experiments carried out in this laboratory (McCarrison and Sankaran, 1933, 1934) it was observed that the growth of various embryonic tissues in plasma taken from fowls suffering from polyneuritis induced by a diet of polished rice is inferior to that of similar tissues in the plasma of birds fed on a satisfactory diet of mixed grains The impairment of growth was particularly marked in the case of nervous tissue and intestinal epithelium In the present series of experiments, the growth of embryonic nervous tissues in plasma taken from rats and fowls on vitamin A deficient diets was studied A number of workers have demonstrated the existence of nervous lesions in vitamin A deficient animals, and it was felt that the investigation might indirectly throw light on the relation between vitamin A and the integrity of nervous tissues

TECHNIQUE

1 *Material for culture*

Fragments of embryonic spinal cord and cerebrum, $\frac{1}{2}$ to 1 square mm in size, were used for culture In the case of the fowl, the spinal cord of an incubated embryo of 7 to 9 days was employed It was found that the nervous tissue of younger or older embryos did not grow so well in culture Ingerbregsten (1913) has made the same observation, and has recorded a similar period of optimum growth for the embryonic nervous tissue of cats, rabbits and dogs

In the early stages of the experiment pregnant rats were taken at random from the stock, and attempts made to culture the nervous tissue of the embryos We found, however, that the best growth occurs when embryos of 15 to 20

(929)

grammes are used   at other stages of embryonic life growth response in culture is difficult to obtain   By placing pairs of male and female rats together for two days and then separating them, we found that this weight, in the Coonoor stock, corresponds with a gestation period of about 19 to 21 days   Subsequently, by similar methods, embryos of about the right age and size were obtained   Three pairs of rats were mated simultaneously to ensure that embryonic   material should be available at the time of experiment, 19 to 21 days later

Cerebral tissue was found to be preferable to spinal cord for tissue culture in the case of the rat , the former shows a more abundant outgrowth of axis cylinders than the latter in suitable and similar cultural conditions   A better growth of axis cylinders can be obtained from rat cerebrum than from fowl cerebrum or spinal cord

2   *Production of vitamin A deficiency*

Eight young fowls, from 1,000 to 1,300 grammes in weight, were placed on the following vitamin A deficient diet, which is a modification of that described by Elvehjem and Neu (1932) —

	Parts
Milled rice	65
Ground whole rice	. 15
Casein	12
Dried yeast	5
Common salt	1
Calcium carbonate	1
Acid calcium phosphate	1

A control group of 8 birds were fed on a diet of similar composition, except that 3 parts of milled rice were replaced by 3 parts of cod-liver oil   In about 8 weeks' time some of the birds on the deficient diet developed leg weakness, which disappeared after they had been exposed to sunlight for a few hours every day for 1 to 2 weeks   It was ascribed to insufficiency of vitamin D, and from the time of its appearance the birds on the diet lacking cod-liver oil were put out of doors for some hours each morning   No recurrence of leg weakness was observed   The earliest symptom suggestive of vitamin A deficiency to appear was a drying up of the comb, which became darker in colour , this was seen in some birds in 4 to 6 weeks Subsequent histological examination revealed that an epithelial change characteristic of vitamin A deficiency—excessive keratinization—was present in these combs At a later period some of the birds showed conjunctivitis and slight cloudiness of the cornea

Blood was taken from vitamin A deficient and control fowls after they had been fed for periods varying from 4 to 16 weeks on the experimental diets (*see* Table) Post-mortem examinations of the vitamin A deficient group revealed no striking macroscopic changes in the internal organs, except in the case of one bird which had advanced pyonephrosis   With 2 exceptions the livers of the vitamin A deficient group gave a negative arsenic trichloride test for vitamin A , the livers giving a positive reaction were subsequently found, by spectrographic assay, to contain only 7γ and 12γ of vitamin A respectively per gramme, amounts far below those usually found in normally fed animals (200γ to 800γ per gramme).

The livers of the control group all gave a strong positive arsenic trichloride reaction, and the general condition of the group was better than that of the deficient group, the average weight at the time of bleeding being about 1,600 g in the former as compared with 1,450 g in the latter

The vitamin A deficient diet used in the rat experiments consisted of the following starch 60 parts, casein 20 parts, olive oil 10 parts, dried yeast 5 parts, salt mixture 5 parts The control diet was similar except that 2 parts of cod-liver oil were substituted for 2 parts of olive oil Twelve animals weighing about 200 g to 250 g were placed on deficient and control diets respectively, and a group of smaller animals, 50 g to 60 g in weight, was similarly dealt with (12 deficient, 5 control) The first group was made up of larger animals because we anticipated difficulty in obtaining blood from smaller animals Subsequent experience, however, showed that comparatively small animals can be used for the taking of blood in the manner described in Section 3

Even after as long a period of 4 months rats on the deficient diet failed to develop xerophthalmia, and post-mortem examination revealed no striking changes They all, however, ceased to grow or lost weight Blood was taken from animals which had been on the experimental diets for 12 to 16 weeks, the more sickly-looking animals in the vitamin A deficient group being usually chosen for bleeding In every case the livers of the vitamin A deficient rats from which blood was taken were free from vitamin A, while those of the control group showed a positive reaction

The vitamin A deficient fowls and rats which were chosen for the withdrawal of blood are listed in the Table Each bleeding was duplicated on a control animal which had been for a similar period on the same diet with the addition of cod-liver oil The livers of these animals showed a positive arsenic trichloride reaction

TABLE

Vitamin A deficient rats and fowls used as source of plasma

Number	FOWLS			Number	RATS		
	Experiment begun	Date of bleeding	AsCl₃ test for vit A		Experiment begun	Date of bleeding	AsCl₃ test for vit A
1	20-7-35	19-8-35	Negative	1	1-6-35	20-8-35	Negative
2	20-7-35	3-10-35	,,	2	1-6-35	9-9-35	,,
3	10-8-35	4-11-35	Positive*	3	1-6-35	21-9-35	,,
4	10-8-35	28-11-35	Negative	4	1-6-35	10-10-35	,,
5	10-8-35	9-12-35	Positive†	5	24-8-35	20-11-35	,,
				6	24-8-35	25-11-35	,,

* 7γ of vitamin A present per gramme of liver.
† 12γ ,,   ,,   ,,   ,,   ,,   ,,   ,,   ,,

3   *Methods of obtaining plasma*

It is comparatively simple to draw 50 c c of blood or more from a fowl by cannulating the carotid artery under light anæsthesia as described by Strangeways (1924)   In rats the superficial arteries are too small for cannulation, and it was found impossible to obtain sufficient blood for our purpose by hypodermic needle from the heart   Accordingly, we were obliged to develop a new technique for obtaining and using rat plasma for tissue-culture experiments, which, since it may prove useful to other workers, will be described in detail *

Blood is obtained by cannulating the abdominal aorta   The rat is secured on its back on a wooden board by stout strings attached to its limbs and by a fine string hooking its incisor teeth   Under ether anæsthesia the skin is removed from the abdominal wall and the muscular coat painted with tincture of iodine   A sterilized towel with a longitudinal slit is spread over the animal and the muscular wall of the abdomen cut away   With fresh sterilized forceps the abdominal aorta and vena cava are exposed   The next step in the operation—the dissection of about half an inch of the aorta from the surrounding tissue and the closely adjacent vena cava—is the most difficult one   Loose areolar tissue can be peeled off with a fine forceps, but the vein lies very near, and frequently medium-sized venous branches cross the artery   If the thin wall of the vein or one of its tributaries is cut, copious bleeding occurs which cannot be stanched, and the animal must be discarded   With a little practice, however, the artery can be held by a blunt forceps and separated from the vein by gentle blunt dissection   The artery is ligatured at the distal end of its separated portion, and on the cardiac end a one inch bull-dog pattern forceps is placed   The wall of the artery is snipped with sterile scissors and a cannula inserted

The cannulæ employed are of the usual Barcroft type, but sufficiently fine to be introduced into a very narrow artery, their tips are about 1 mm to 1 5 mm in diameter   Such cannulæ are easily made with a little practice About 6 of these, of varying sizes, sterilized in olive oil, are kept at hand during the operation   When the appropriate cannula (the bigger the cannula which can be used the better) has been inserted, it is tied in position   On releasing the forceps the blood flows freely through in a steady stream   The first few drops of blood are discarded, since it was found that a tube of blood which includes the first drops readily clots, possibly because these drops contain thrombokinase liberated by the traumatic action of snipping the artery and introducing the cannula

In this manner as much as 20 c c of blood can be collected   Blood is drawn into 4 or 5 narrow centrifuge tubes, paraffin coated and ice cold   The collected blood is immediately centrifuged in ice packing and the plasma, subsequently drawn off with cold paraffined pipettes into fresh paraffined cold tubes   When the blood has been taken the animal is killed

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*After this paper had been submitted for publication, we received the ' *Archiv für experimentelle Zellforschung* ', 1935, **18,** No 2, in which Zechel (p 142) describes a somewhat similar technique for obtaining rat plasma   In Zechel's technique, Heparin is used to prevent clotting, which would complicate tissue culture experiments with a nutritional bearing.

4 *Clotting of plasma*

The plasma of normal fowls will remain unclotted, and thus suitable for tissue culture experiments, for days or weeks in a Frigidaire Rat plasma, collected by the method described, clots very soon even at 0°C , and must be used for implantation of tissue within an hour of being drawn It is, therefore, necessary to have the tissue fragments previously cut and ready for mounting on slides before blood is taken In mounting the cultures, it is strictly necessary that the pipette used for taking up plasma should be kept in a wide glass tube surrounded by ice , neglect of this precaution results in the clotting of the plasma after a few slides have been put up In spite of all precautions, however, rat plasma will clot within an hour and there is no possibility of using it for a second tissue-culture experiment Clotting, both in the fowl and the rat, appears to be hastened by vitamin A deficiency Of the 6 specimens of 'deficient' fowl plasma obtained, only one remained fluid for 6 days, and the remainder clotted within 48 hours

5 *Implantation in plasma and Tyrode solution*

Cultures were grown in 3 types of plasma obtained from fowls and rats (*a*) on the vitamin A deficient diet, (*b*) on the same diet plus cod-liver oil and (*c*) on a good mixed diet (mixed grains for the fowls and the Coonoor stock diet for the rats) Fragments for incubation were implanted in a mixture of equal parts of plasma and Tyrode* solution on coverslips, inverted over hollow ground microscope slides, and sealed with paraffin Clotting takes place in the incubator All operations were carried out under strictly sterile conditions

The pre-incubation stage of a single (rat) culture experiment may be summarized as follows first, the nerve tissue of a suitable embryo is fragmented , next, the plasma of 3 rats (deficient, control, and stock) is obtained , finally, the tissues are implanted within an hour The procedure in the case of the fowl is similar, except that there is not the same urgency to implant rapidly after the withdrawal of blood.

THE GROWTH OF CULTURES

Suitable fowl spinal cord and rat cerebrum cultures were found to grow well in Tyrode and plasma obtained from the animals fed on a good mixed diet Within 6 hours one can observe the outgrowth of filaments with a bulbous end which in some instances puts out a few tiny branches This branching is more commonly observed in rat than in fowl tissue The filaments, which are in fact axis cylinders, can be traced back to a nerve cell in the explant As time passes they steadily elongate, and the rate of elongation can easily be watched through a microscope placed inside an incubator which keeps the cultures at 38 4°C It was found to take place uniformly at the rate of 4 8 to 6 0 microns per hour After about 60 hours the axis cylinders cease to elongate, and at the same time beading occurs throughout their length—a phenomenon perhaps akin to Wallerian degeneration. Even when this point is reached, a few new filaments may be observed growing out

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* Tyrode solution is NaCl—0 8 per cent, KCl—0 02 per cent, CaCl$_2$—0 02 per cent, NaH$_2$PO$_4$— 0 006 per cent, NaHCO$_3$—0 05 per cent, glucose—0 1 per cent in double glass distilled water

of the explant   After about 72 hours the beaded axis cylinders undergo autolysis and disappear

There was a very definite difference between cultures in ' deficient ' plasma and those in ' normal ' plasma   In the former, axis cylinders were more scanty, and those which appeared did not grow to the same length as in the latter   Degeneration of axis cylinders, shown by beading, and autolysis took place in a shorter time—as early as 48 hours

Growth of axis cylinders in the ' control ' plasma, while quite abundant and superior to growth in ' deficient ' plasma, did not equal that in ' normal ' plasma

We carried out in all 11 culture experiments, 6 with rat plasma and 5 with fowl plasma   In each experiment about 6 explants were seeded in each type of plasma   On examination of cultures after 24 hours' incubation, it was always easy to observe a difference in growth between ' normal ' and ' deficient ' cultures   Since it was difficult to express this difference quantitatively, and self-deception is easy, we made a number of attempts to group slides with ' good ', ' medium ' and ' poor ' growth, while remaining in ignorance of the medium in each case   As criteria of growth, abundance and length of axis cylinders, and absence of degeneration, were employed   The results of one attempt at ' blind ' selection was as follows —

Good growth		Medium growth		Poor growth	
' Normal ' plasma	5	' Control ' plasma	1	' Deficient ' plasma	5
' Control '	1	' Normal	1	' Control '	1

It will be observed that in this case all the ' deficient ' plasma slides were placed in the ' poor ' group   In other experiments we (and other workers in the laboratory) were able to group slides with equal accuracy, and convince ourselves that the differences observed were not due to suggestion

Microphotographs of a few stained cultures are given (Plate XL, figs 1 to 6)   They were fixed for 48 hours in 10 per cent neutral formalin and stained with Ehrlich's acid hæmatoxylin

DISCUSSION

In a previous experiment (McCarrison and Sankaran, 1933) it was observed that subnormal nerve tissue growth occurred in the plasma of birds suffering from ' polyneuritis '   Polyneuritis was induced by a diet of polished rice, which is deficient in vitamin A as well as in vitamin B_1 It is therefore possible that the phenomena previously reported were essentially similar in nature to those now described

A number of workers have observed nerve degeneration in vitamin A deficient animals   The literature of this subject has recently been reviewed by Sutton, Setterfield and Kraus (1934)   Pathological investigations on vitamin A deficient rabbits and fowls in this laboratory have revealed advanced stages of nerve degeneration   Mellanby (1934) has suggested that the epithelial changes characteristic of vitamin A deficiency are secondary to trophic nerve lesions   It seems reasonable to suppose that the phenomena observed in our experiments are parallel

PLATE XL
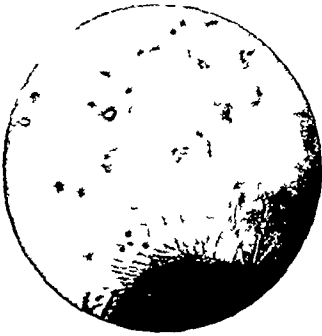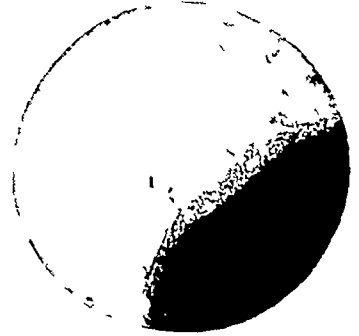
FIG 1   FIG 2   FIG 3

Photomicrographs of living unstained cultures of rat embryo cerebral tissue, 24 hours' growth in 'normal' (Fig 1), 'control' (Fig 2), and 'deficient' (Fig 3) rat plasma and Tyrode solution
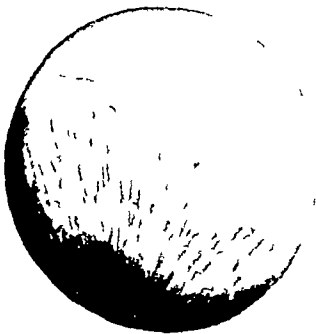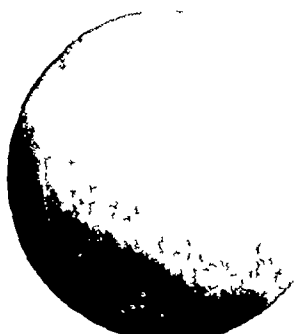
FIG. 4   FIG 5   FIG 6

Photomicrographs of stained cultures of chick embryo spinal cord tissue, 24 hours' growth in 'normal' (Fig 4), 'control' (Fig 5), and 'deficient' (Fig. 6) fowl plasma and Tyrode solution (Ehrlich's acid hæmatoxylin)

to those which occur in the nerves of vitamin A deficient animals We must, however, underline the fact that growth in 'control' plasma did not equal that in 'normal' plasma The condition of animals on synthetic diets supplemented by vitamin rich materials (dried yeast, cod-liver oil, etc) and mineral salts is never quite as good as that of animals on a good mixed diet, a sub-optimal 'state of nutrition' is present, which in our experiments expressed itself in the manner described

No conclusion can be drawn as to the reason why the plasma from the deficient animals failed to support good growth of nerve cells in culture It is possible that in our experiments there was an actual deficiency of vitamin A in the plasma We have however, so far been unable, by spectrographic methods, to detect vitamin A or carotene in any kind of rat or fowl plasma On the other hand, various changes in plasma probably associated with vitamin A deficiency have been described, e g, a decrease in blood serum esterase (Green, 1934), a decrease in blood lipase (Holm, 1934) Any of these may conceivably be inimical to the growth of nerve cells in culture The fact that growth was subnormal in 'control' plasma taken from animals in a sub-optimal 'state of nutrition' suggests that the impairment of growth in 'deficient' plasma was due to an alteration in the state of the plasma rather than to an absence of vitamin A

The observation that the growth of embryo cells in the plasma of an animal in a poor 'state of nutrition' is subnormal seems of some general interest The growing cells of the embryo are ultimately dependent on the plasma of the mother for their nourishment It seems likely that if her diet is deficient in vitamin A, fully normal development of embryonic brain and other tissue can not take place

SUMMARY

1 Embryonic nerve tissue was cultured in plasma from fowls and rats on (*a*) a good mixed diet, (*b*) a diet deficient in vitamin A and (*c*) a control diet similar to (*b*) except that it contained cod-liver oil

2 Excellent growth took place in 'normal' plasma and poor growth in 'deficient' plasma In 'control' plasma the growth response lay between these extremes

3 A technique of obtaining rat plasma in sufficient quantity for tissue culture experiments is described in detail

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THE CAROTENE CONTENT OF SOME INDIAN VEGETABLE FOOD-STUFFS WITH A PRELIMINARY NOTE ON ITS VARIATION DUE TO STORAGE

Parts I—II.

BY

N K DE

(From the Nutrition Research Laboratories, I R F A , Coonoor)

Part I.

It has been abundantly proved that carotene, the yellow lipochrome pigment of plant materials, can fulfil the physiological functions of vitamin A, transformation of carotene into vitamin A taking place *in vivo* (Moore, 1930)   The vitamin A activity of vegetable food-stuffs appears to be due solely to their carotene content (Wolf *et al*, 1930, Moore, 1931, Guilbert, 1934, De, 1935)   Until a few years ago vitamin A activity was estimated solely by the biological test, and few systematic investigations of the carotene content of food-stuffs, in India and elsewhere, have as yet been carried out   Recently, Kuhn and Lederer (1931), Kuhn and Brockmann (1933) and others have established the fact that carotene may exist in different isomeric states (α, β and γ forms), the β isomer being twice as potent as either the α or   isomer   The total amount of carotene present in a food-stuff may, however, be a valuable indication of its importance as a source of vitamin A   Accordingly, the author has estimated the (total) carotene content of some 80 vegetable food-stuffs, using a simplified spectrophotometric method previously described (De, 1935)   The investigation is a part of the systematic survey of Indian food-stuffs at present in progress in these laboratories

EXTRACTION OF CAROTENE FROM THE TEST MATERIALS

The investigation covered green leafy vegetables, roots, fruits, legumes and cereals   Unless otherwise stated in Table I, the samples were obtained from the local market, they were in the state in which they are commonly bought and consumed, but had not the freshness of vegetables just plucked or harvested   Where the food-stuffs are stated to be fresh, it is to be understood that the specimens were analysed within a few hours of obtaining them from vegetable gardens close

(937)

by   The amount of a food-stuff taken for analysis lay between 2 g and 50 g, and varied according to the nature of the substance   The material (only the edible portion, unless otherwise stated) was finely ground in a porcelain mortar with a little glass powder and $CaCO_3$, extracted thrice with absolute alcohol and then 3 to 5 times with petroleum ether or carbon-bisulphide (CS_2) until no pigment was visible in fresh solvent   The alcohol and petroleum ether (or CS_2) extracts were collected together in a separating funnel and a few c c of distilled water added to make the alcohol to about 85 per cent   On shaking, carotene was taken up by petroleum ether (or by CS_2), while the irrelevant pigments (part of the chlorophylls, xanthophylls, rhodoxanthin, fucoxanthin etc) remained in the alcohol   The carotene solution was then washed successively with 85 per cent and 70 per cent alcohol, and repeatedly with water   The solution was next freed from moisture by treating with a little anhydrous $CaSO_4$, and shaken with sufficient quantity of finely powdered and dry $CaCO_3$ to remove chlorophylls and the possible traces of the non-hydrocarbon pigments (Palmer, 1922)   The filtrate was made up to suitable volume and its carotene content assayed spectrographically   Whenever the examination could not be carried out immediately, the solution was preserved in the dark with a little hydroquinone to prevent oxidation

ESTIMATION OF CAROTENE FROM THE EXTRACTS

The method was essentially that described previously (De, 1935)   As a result of experience, however, the usual spectrophotometric technique was modified in several respects   The short ultra-violet radiations from the source may sometimes appreciably bleach the pigment, even during the short time (5 to 10 minutes) required for taking an absorption photograph   The deterioration was found to be very marked in $CHCl_3$, the only solvent in which spectrophotometric estimations of carotene are usually carried out   Even under ordinary conditions chloroform tends to destroy the pigment more quickly than the other solvents   In the present investigation the short ultra-violet radiations of the source were cut off by filtering the light through a thick glass plate, and, instead of applying the usual procedure—namely, drying up the extracts and examining the chloroform solution of the residue—the petroleum ether or CS_2 extracts were spectrographed directly   The absorption cell was filled with the extract (in suitable concentration), while the compensating cell was filled with a solution from which carotene has been completely removed by irradiation (De, 1935), as evidenced by absence of colour or by a negative arsenic trichloride test   Even after complete removal of carotene, CS_2 solutions may sometimes exhibit a non-interfering colour, due to dissolved sulphur compounds formed during irradiation   Experience showed that the use of the CS_2 solvent should, whenever possible, be avoided as it has an irritating pungent smell and requires special attention during irradiation

An absorption photograph was taken for each test solution and the value of the extinction coefficient at the region of maximum absorption (near 4500 and 4800 A U for petroleum ether and CS_2 solutions of carotene respectively) determined   For calculation of the carotene content the following formulæ were used

$$E\frac{1\ \text{per cent}}{1\ \text{cm}} = 1,600, \text{ for } CS_2 \text{ solutions (De, 1936) and } E\frac{1\ \text{per cent}}{1\ \text{cm}} = 2,500 \text{ for}$$

the petroleum ether solutions of carotene (Gillam, 1935 De, 1936)

A colorimetric method of assaying carotene was applied in a few cases, it proved however, unsatisfactory for two reasons the standard potassium bichromate solution did not obey dilution law, and the colour of the final test solutions often differed from that of the bichromate (or carotene) standards A few colorimetric estimations were made with extracts having identical colour, using a pure carotene solution as standard of comparison The figures agreed closely with those obtained by the spectrophotometric method Carotene standards for use in quick estimations should be preserved in the dark, by adding a little hydroquinone to prevent oxidation, and their concentration should be checked spectrographically now and then For the estimation of carotene in lycopene rich materials, e g, ripe tomatoes, red chillies, etc, the procedure described in an accompanying communication (De, 1936) was adopted In the case of these foods, figures must be regarded as approximate

By applying the above spectrophotometric technique, as many as 3 to 5 estimations could be carried out in a day and with experience 2 to 4 extracts could be photographed on a single plate The results are set out in Table I —

TABLE I

Carotene content of various vegetable food-stuffs

Food stuffs	Carotene content in γ (0 001 mg) per g or c c of material as obtained
VEGETABLES	
Amaranth (large leafed variety) (*Amaranthus gangeticus*) fresh matured green leaves	111 00
Amaranth (small leafed variety) (*Amaranthus gangeticus*), fresh, matured green leaves	75 34
Amaranth (small leafed variety) (*Amaranthus gangeticus*), very tender	25 20
Betel leaves (*Piper betel*)	72 70
Bitter gourd (*Momordica charantia*)	2 10
Brinjal (*Solanum melongena*)	0 05
Cabbage, outermost green leaf, fresh	39 27
„ outer green leaf, fresh	31 60
„ inner green leaf, „	23 80
„ inner white leaf, „	0 90
„ innermost white portion, fresh	0 32

TABLE I—*contd*

Food stuffs	Carotene content in γ (0 001 mg) per g or c c of material as obtained
VEGETABLES—*concld*	
Cauliflower	0 38
Celery leaves	57 60
Coriander leaves, fresh (*Coriandrum sativum*)	126 30
Chillies (green), long variety (*Capsicum annuum*)	2 63
„ („), round and short (*Capsicum frutescens*)	3 60
„ („), different sample, round and short	7 40
„ ripe, dry (*Capsicum annuum*)	5 76
Curry leaves (*Murraya lœnigu*)	126 30
Drumstick (*Moringa oleifera*)	1 84
French bean (*Phaseolus vulgaris*)	2 21
Garlic	Nil
Green peas	1 39
Ladies' finger (*Hibiscus esculentus*)	0 58
Lettuce, fresh tender leaves	20 60
„ , leaves, different sample	24 00
Neem leaves, tender (*Azedirachta indica*)	45 60
Onion, small variety	0 25
Parsley, fresh	32 00
Pumpkin, sweet variety (*Cucurbita maxima*)	0 84
Ridge gourd (*Luffa acutangula*)	0 56
Snake gourd (*Trichosanthes anguina*)	1 60
Spinach, fresh tender leaves	26 30
Tea leaves, dry dust (Nilgiris)	25 40
TUBERS AND ROOT VEGETABLES	
Carrot, fresh tender	20 20
Colocasia (*Colocasia antiquorum*)	0 40

TABLE I—*contd*

Food stuffs	Carotene content in γ (0 001 mg) per g or c c of material as obtained
TUBERS AND ROOT VEGETABLES—*concld*	
Parsnip	0 30
Potato, white, poorly manured	*Nil* or trace
,, ,, manured	0 56
Radish	0 03
Turnip, white	*Nil* or trace
Yam	4 34
FRUITS	
Apple (Australian)	*Nil* or trace
Coco nut (kernel)	*Nil* or trace
Grape juice, preserved	0 15
Lime (matured), juice only	0 26
,, skin only	3 05
Mango, green fruit	1 50
Orange (green skin, ripe fruit), juice	4 20
,, (different fruit), juice only	3 70
,, (,, ,,), skin only	25 70
Papaya, green matured fruit (*Carica papaya*)	2 40
,, ripe fruit	18 40
Pears, country, partly ripe	0 14
Plantain, green, used as vegetables	0 50
,, different sample	0 46
,, ripe fruit	1 24
Tomato (green tender fruit), juice only	0 25
,, (,, matured ,,), ,, ,,	0 53
,, (,,), whole fruit	3 20
,, (ripe fruit), juice only	5 70

TABLE I—*contd*

Food stuffs	Carotene content in γ (0 001 mg) per g or c c of material as obtained
FRUITS—*concld*	
Tomato juice, deep red, preserved (Canadian)	11 00
„ „ light „ , (S African)	5 40
CEREALS, PULSES AND LEGUMES	
Bengal gram, with husk (*Cicer arietinum*)	4 16
Black „ without husk (*Phascolus mungo*)	0 64
Cambu (*Pennisetum typhoideum*)	2 20
Cholam (*Sorghum vulgare*)	1 60
„ from Bellary (*Sorghum cernuum*)	2 10
„ (collected by Coimbatore Agriculture College)	1 80
, yellow (*Sorghum durra*) (collected by Coimbatore Agriculture College)	0 70
, yellow Jonni seeds ' (*Sorghum cernuum*) (collected by Coimbatore Agriculture College)	0 57
Green gram, with husk (*Phascolus radiatus*)	1 58
Horse „ „ „ (*Dolichos biflorus*)	1 58
Maize, raw white	0 42
Mussoor dhal (lentils), without husk (*Lens esculenta*)	4 50
Ragi sample 1 brown variety (*Eleusine coracana*) (collected by Coimbatore Agriculture College)	0 83
sample 2, brown variety (*Eleusine coracana*) (collected by Coimbatore Agriculture College)	0 90
sample 3, brown variety (*Eleusine coracana*) (collected by Coimbatore Agriculture College)	0 37
Red gram (without husk) (*Cajanus indicus*)	2 20
Rice, white, polished	*Nil*
„ (Mangalore), unpolished	0 19
, („) slightly polished	0 16
„ („), polished to some extent	0 12
Soya bean small size variety (with husk)	4 50
„ „ large „ (from Charodi Farm, B P)	9 70
Wheat, whole (Punjab)	1 08
VEGETABLE OILS AND SEEDS	
Coco nut oil	*Nil* or trace
Coriander seeds (*Coriandrum sativum*)	15 70
Gingelly oil (*Sesamum indicum*)	*Nil*
„ seeds	1 07

TABLE I—*concld*

Food stuffs	Carotene content in γ (0 001 mg) per g or c c of material as obtained
VEGETABLE OILS AND SEEDS—*concld*	
Ground nut oil (*Arachis hypogœa*)	Nil or trace
„ seeds	0 63
Mustard oil (*Brassica juncea*)	Nil
„ seeds	2 70
Olive oil	Nil
Red palm oil (Malaya) (*Elœis guineensis*)	400 00
MISCELLANEOUS	
Cumin (*Cuminum cyminum*)	8 70
Jaggery, black, not refined (from sugar cane juice)	2 80
„ brown, partly refined („ „ , ,)	0 14
Sago	Nil
Tamarind (*Tamarindus indica*)	0 86
„ different sample	1 10
Turmeric (*Curcuma longa*)	0 30
Yeast (dried brewer's)	1 10

One International Unit of vitamin A has been defined as the vitamin A activity of 0 6γ of pure β carotene Considering the heterogeneous nature of carotene in food stuffs, one International Unit may be taken as being about equal to 1γ of carotene as estimated in this investigation An average biological unit has roughly the same value; on this basis of comparison, there is fair agree ment between the figures given here and available figures obtained by biological method (Sherman and Smith, 1931)

DISCUSSION AND RESULTS

Green, leafy vegetables in general are rich sources of carotene Amaranth, spinach, green cabbage leaves, lettuce, coriander leaves, betel leaves, neem leaves, etc , all contain large quantities of carotene Bitter gourd, snake gourd, French beans, drumstick, green peas and pumpkin, etc , are fairly good sources of carotene Among the various root vegetables examined, carrot was found to be very rich, yam was fairly good, while potato, colocasia, turnip, parsnip and radish were poor Of the fruits examined, the ripe ones were richer in carotene than the green ones Papaya, plantain, orange and tomato, when ripe, become good sources of carotene. Of the various cereals and legumes analysed, soya bean, mussoor dhal, green gram, Bengal gram, etc , are good sources , cambu, cholam, ragi and whole wheat, though not rich, are better sources of carotene than rice Polished rice was found to be devoid of carotene, while the unpolished samples contained traces It was noted

that refined jaggery had a lower content than crude samples Carotene was not detected in any of the vegetable oils examined, with the exception of red-palm oil It is interesting to note that red-palm oil (carotene content $= 400\ 0\ \gamma$ per g) stands pre-eminent in the list of the food-stuffs analysed

It was observed that the carotene content of the non-leafy vegetables did not always run parallel to their yellow coloration, e g , most of the yellow pigment in turmeric is water or alcohol soluble, carotene being present in negligible quantity Green leaves are very rich compared to white ones The carotene content of the outer green leaves of cabbage was found to be as much as 40 times that of the inner white portion Similar wide variations were observed by the biological test in the vitamin A activity of green and white leaves of cabbage, lettuce and spinach, etc (Sherman and Smith, *loc cit*) Similarly, carotene is found in much larger quantities in the skin of oranges and limes than in their juice or inside portions These observations corroborate the general view that photochemical activity plays an important rôle in the synthesis of carotene in plants

It is noteworthy that a number of the vegetable food-stuffs tested have a carotene content which approximates to or exceeds the vitamin A content of milk fat Carotene-rich vegetable foods, which in general are much cheaper and more easily available than animal fats, would seem to be of great importance in a country like India At present, however, we have little knowledge as to relative value of equal quantities of vitamin A and carotene in the diet of human beings

Part II.

Effect of Storage on the Carotene Content of Some Vegetable Food-Stuffs

Simultaneously with the carotene assay work reported in Part I, a few observations were made on the effect of storage on the carotene content of some vegetable food-stuffs Pure carotene is known to be very unstable and liable to destruction by light and atmospheric oxidation As most food-stuffs are not usually consumed in a state of absolute freshness but have lain in shops and markets for hours and in some cases for days and months before purchase and consumption, a knowledge of the variation of carotene content on storage under ordinary conditions (e g , without preservatives) is desirable Further, a point of interest has been raised by the recent report of MacLeod and his associates (1935) who, conducting some biological vitamin A experiments, noted a strange result, namely, that the vitamin A potency of some varieties of sweet potatoes increased several times after storage for two months or more at room temperature To explain this observation they have suggested that either an absolute increase of carotene takes place, or that existing carotene may become more assimilable to the animal system as a result of storage

The materials examined in the present investigation were preserved in baskets or glass jars, at various temperatures, under ordinary conditions The carotene content was estimated at the stated periods of storage by applying the method described in Part I, values being given per gramme or c c of initial weight Sweet potato was not available in Coonoor during the season when the investigation was carried out The observed data are set out in Table II —

TABLE II.

Variation of the carotene content of some food-stuffs during storage at various temperatures

Temperature of storage	Food	Initial carotene content in γ per g or c.c.	Carotene content in γ per g or c.c. of initial weight at the quoted periods of storage				Condition of the material when it was finally examined
0°C	Amaranth leaves	111.00	67.1 (6th day)				Unfit for consumption
Room temperature 16°C to 22°C	Amaranth leaves	111.00	62.2 (6th day)				Unfit for consumption
	Bengal gram	3.16				6.8 (59th day)	Still in good condition
	Chillies, green	3.60	7.4 (7th day)	9.25 (12th day)			Dried up and developed red colour
	Cholam	1.60				0.99 (60th day)	Condition not good
	Colocasia	0.40		0.71 (21st day)	0.42 (41st day)	0.17 (70th day)	Still in good condition
	Green gram	1.58		5.3 (46th day)	13.0 (79th day)	1.27 (134th day)	Still in good condition
	Orange (green skin), juice	3.70		4.2 (12th day)	1.11 (34th day)		Unfit for consumption, skin turned yellowish.
	Parsnip	0.30	0.58 (7th day)	0.69 (27th day)			Dried up and unfit for consumption
	Potato	Nil or trace	0.31 (33rd day)	0.37 (60th day)	0.17 (90th day)	0.14 (120th day)	Condition good sprouted

TABLE II—*concld*

Temperature of storage	Food	Initial carotene content in γ per g or c c	Carotene content in γ per g or c c of initial weight at the quoted periods of storage					Condition of the material when it was finally examined
Room temperature 16°C to 22°C	Radish	0 00			0 04 (11th day)	0 06 (27th day)	0 09 (34th day)	Unfit for consumption
	Red gram	2 2				7 4 (61st day)	1 25 (128th day)	Still in good condition
	Tomato (green fresh) juice	0 51	0 05 (9th day)	2 50 (15th day)				Green fruits ripened to red colour
	Turnip	Nil or trace		0 11 (18th day)				Unfit for consumption
	Wheat, whole	8 00						
	Yam	4 54			7 4 (52nd day)	0 74 (60th day)	10 5 (73rd day)	Condition not good Putrefied
37°C	Amaranth leaves	111 00	72 15 (2nd day)	36 9 (3rd day)				Unfit for consumption
	Chillies, green	3 60	11 1 (5th day)	2 24 (8th day)	0 63 (10th day)			Putrefied No red or yellowish colour developed
	Orange (green skin) juice	4 20	5 3 (5th day)	5 9 (8th day)	1 6 (10th day)			Condition not good, skin turned yellow
	Plantain, green	0 46	0 49 (5th day)	0 40 (8th day)	0 24 (11th day)			Green fruits ripened and putrefied
	Tomato (green) juice	0 53	2 7 (5th day)	2 0 (8th day)	1 6 (10th day)	0 83 (15th day)		Green fruits ripened with yellow colour condition not good

The specimen of amaranth leaves analysed lost 37 per cent and 44 per cent of carotene after storage for 6 days at 0°C and 20°C (room temperature) respectively, while after only two days' storage at 37°C, the corresponding loss was about 67 per cent   The cereals examined, cholam and wheat, lost 40 per cent and 30 per cent of carotene respectively in 2 months' storage at the room temperature

The pulses, roots and fruits investigated showed a steady *increase* (1 to 8) of carotene content, during varying, and in some cases prolonged, periods of storage after a certain point was reached comparatively rapid deterioration of carotene occurred   Green gram showed steady increase (1 to 8) even up to the 79th day White potato showed increase of carotene during 2 months of storage, after which deterioration commenced   This development of carotene followed by deterioration seemed to take place more rapidly at higher temperatures, and the upper limit to which carotene formation proceeds appeared to be considerably lowered at such temperatures (note the figures for orange juice)   The rate of the development of carotene and of its deterioration in any given food-stuff during storage will probably vary according to its state of maturity

It is interesting to note that the carotene content of some common root vegetables (radish, turnip parsnip, etc), and of fruits like oranges, preserved under mild or cold climatic conditions may steadily increase until the point at which the materials become putrefied and unfit for consumption

In conformity with Duggar's (1913) findings, the interesting fact was noted that green tomatoes preserved at the room temperature reddened on ripening, while those preserved at 37°C ripened with a yellow colour   When some of the yellow fruits were returned (from 37°C) to storage at room temperature, they turned red, rapidly   It is probable that the higher temperature suppresses the formation of the red pigment lycopene   Further studies on this point are in progress

SUMMARY

1   The carotene content of some 80 Indian vegetable food-stuffs has been estimated using a modified spectrophotometric method

2   Development of carotene, consequent on storage, was observed to take place in some non-leafy vegetable foods, such as roots, pulses and fruits   Synthesis of carotene appears to continue until long after harvesting or plucking   Ultimately deterioration occurs

3   The carotene content of some non-leafy vegetables which are generally consumed fresh did not appreciably alter during a reasonable period of storage under ordinary conditions

4   A leafy vegetable (amaranth) showed a marked loss of carotene after one or two days' storage, the loss being more rapid at higher temperatures

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A COMPARATIVE STUDY OF SOME PROPERTIES OF CAROTENE AND LYCOPENE

BY

N K DE

(*From the Nutrition Research Laboratories, I R F A , Coonoor*)

THE systematic assay of carotene in food-stuffs has become an important problem in nutrition, since carotene can fulfil the physiological functions of vitamin A (Moore, 1930)   The methods usually employed for the isolation and estimation of carotene are not, however, fully satisfactory   By applying the phase tests between petroleum ether or carbon-disulphide (CS_2) and alcohol of suitable strengths, and by adsorption on $CaCO_3$, the active hydrocarbon pigment carotene can be isolated from the associated inactive non-hydrocarbon lipochromes (chlorophylls, xanthophylls, rhodoxanthin, fucoxanthin, etc)   No satisfactory method, however, has yet been devised for separating the active factor from its inactive red isomer lycopene   Hence, difficulties are encountered in the analysis of substances which contain an appreciable amount of lycopene, e g , ripe tomatoes, red chillies and some varieties of fruits and vegetables   The only method of effecting separation in such cases is to resort to repeated fractional crystallization, made possible by the fact that lycopene is less soluble than carotene in all the carotenoid solvents   Such a costly, lengthy and tedious method can be of little use in routine work   The characteristic properties of the two pigments, hitherto studied, which may be serviceable in distinguishing them, are differences in colour, solubility and position of absorption bands (Palmer, 1922)   Solutions of lycopene have a somewhat brown tone in comparison with similar solutions of carotene CS_2 solutions of lycopene are characterized by their bluish red colour which persists even in great dilution, while solutions of carotene, in the same solvent, change to a yellowish red colour   Both show two or more absorption bands in the visible region of the spectrum, the lycopene bands, compared to those of carotene, lying towards the red end   The positions of the absorption bands vary from solvent to solvent and a marked shift between the two series occurs in CS_2 solutions (Monteverde, 1913 , Willstratter, 1913)   It can be expected from the above that mixtures of carotene and lycopene will give different positions of absorption maxima, depending upon the proportions of the

individual pigments, no work, however, has hitherto been carried out on this question Besides the few properties stated above, there exist no other means to differentiate lycopene from carotene The behaviour of the former towards the mineral acids, various adsorbents and reagents has not yet been studied, although some data in the case of carotene are available (Palmer, *loc cit*)

The present investigation was undertaken to compare the action of some reagents and adsorbents on carotene and lycopene, and to observe whether the difference in the positions of their absorption bands can be made use of in developing a technique for estimating the pigments separately when they occur together

PREPARATION OF THE PIGMENTS.

The International Standard Preparation of carotene (1932) was used for all studies made on carotene The specimen consists mainly of β carotene (League of Nations Health Organization, M 20, June 1932) Lycopene was prepared from fully ripe (red-skinned) tomatoes Five fruits weighing about 400 grammes were minced and the juice squeezed out The solid materials were extracted twice with alcohol and thrice with CS_2, the extracts being rejected Since carotene is much more soluble than lycopene, this treatment should remove all or almost all of the carotene present leaving behind only pure lycopene The lycopene was then extracted (with CS_2) from the residue and purified, by the usual procedure applied for carotene It proved impossible, by fractional crystallization, to remove any carotene from the lycopene solution, possibly because the pigments were not present in sufficient quantities Separation of carotene from lycopene, however, was effected by the following procedure, revealed during preliminary studies The CS_2 solution was shaken with an amount of dry and finely powdered MgO, not quite sufficient for complete adsorption, the major portion of the lycopene was adsorbed on the agent, while any carotene present remained with a little lycopene in solution The pigment was dissolved with acetone, dried up under reduced pressure at low temperature, redissolved in CS_2, and the process of fractional adsorption repeated thrice The final solution was made up to 50 c c in CS_2 The solution showed the characteristic bluish red colour of lycopene and appeared to be sufficiently pure, its purity was subsequently verified spectrographically The concentration was, however, much reduced owing to considerable wastage during the processes of purification The solution of lycopene was preserved in the dark (with a little hydroquinone to prevent oxidation) until required for examination

COMPARATIVE STUDIES ON CAROTENE AND LYCOPENE

The actions of a few reagents and adsorbents on the two isomeric hydrocarbons were then studied The observations were only qualitative The solutions of carotene used contained 4.5γ of carotene per c c of CS_2 A part of the prepared lycopene solution was brought to approximately the same strength (4.8γ per c c) by diluting it to a similar depth of colour In order to make a rough comparison between the properties of the two pigments, equal amounts of the reacting materials were added to equal quantities of the individual pigments The results of the

studies on colour reactions are set out in Table I and those of the studies on adsorbents in Table II —

<center>TABLE I</center>

The colour reactions of certain reagents with carotene and lycopene.

	Reagents	State of the pigment	Reaction with carotene	Reaction with lycopene	REMARKS
1	Conc HNO₃	Dry or alcoholic solution	Weak blue colour	Blue colour	
2	Conc H₂SO₄	do	do	do	
3	Con HCl	do	*Nil*	*Nil*	
4	Sulphurous acid (dry)	do	Weak blue colour	Blue colour	Lycopene compared to carotene appeared to show slightly deeper colour reactions
5	Formic acid	do	*Nil*	*Nil*	
6	Phosphoric acid	do	*Nil*	*Nil*	
7	Oxalic acid	do	*Nil*	*Nil*	
8	Ferric chloride (Con solutions)	Dry	Green colour	Green colour	
9	Arsenic trichloride (pure)	do	Blue colour	Blue colour	
10	Antimony trichloride (in CHCl₃) solutions	do	do	do	

TABLE II

The behaviour of carotene and lycopene towards certain adsorbents

Number of observations	Adsorbent	Carotene in CS_2 solution	Lycopene in CS_2 solution	Remarks
1	MgO	Adsorption	Adsorption	Lycopene in general showed greater affinity for adsorption than carotene on all the adsorbents used The adsorption seemed to be accompanied by oxidation in some cases and the destruction of lycopene appeared to be more rapid than that of carotene
2	Al_2O_3 (alumina)	do	do	
3	CaO	do	do	
4	Ca (OH)$_2$	do	do	
5	$CaCl_2$.	do	do	
6	$CaCO_3$	No adsorption	No adsorption	
7	$CaSO_4$	do	do	
8	Ferric sulphate	Adsorption and oxidation	Adsorption and oxidation	
9	Silver acetate	do	do	
10	PbS	do	do	
11	Ba (OH)$_2$	Adsorption	Adsorption	
12	$BaCl_2$.	do	do	
13	Phosphotungstic acid	Adsorption and oxidation	Adsorption and oxidation	
14	$HgCl_2$	do	do	
15	Charcoal	do	do	
16	Silica	Adsorption	Adsorption	

It will appear from the above tables that there is no fundamental difference between the two pigments as regards the properties studied A point of importance was, however, revealed, namely, that lycopene compared to carotene had a greater affinity for adsorption, and showed slightly deeper colour reactions It is interesting to notice that the adsorption affinities of the two pigments are the reverse of their relative solubility, lycopene is less soluble than carotene in all the carotenoid solvents It therefore appears possible to isolate carotene from lycopene by repeated fractional adsorption or chromatography on a suitable adsorbent (Table II) This general conclusion is supported by recent reports of Schon (1935) and Gillam and Heilbron (1935) In a chromatographic study (on Al_2O_3 as adsorbent) of the carotenoid hydrocarbon pigments, extracted from 15 kg of some Portuguese fruits, Schon observed a new zone (red, narrow and sharp), just above the carotene zones This he found spectrographically to be due to lycopene Gillam and Heilbron, conducting similar studies, detected a new pigment, lycopene, in some samples of Danish butter Chromatographic analysis has, however, similar disadvantages to those of fractional crystallization Up to the present, therefore, no satisfactory simple method has been devised for assaying carotene when it occurs simultaneously with lycopene

Variations in the values of $E_{1\,cm}^{1\,per\,cent}$ *(extinction coefficient for a one per cent solution in a one cm cell) for carotene and lycopene in different solvents*

Recently, Gillam (1935) claims to have isolated carotene (β isomer) in a purer form than has hitherto been obtained, this specimen gave $E_{1\,cm}^{1\,per\,cent} = 2,200$ in chloroform and 2,500 in petroleum ether It is thus established that, as the absorption bands of the carotenoid pigments vary from solvent to solvent, so do the values of $E_{1\,cm}^{1\,per\,cent}$ In chloroform solutions lycopene showed $E_{1\,cm}^{1\,per\,cent} = 2,000$ (Gillam, *loc cit*, and Pummerer, 1935) Similar values for carotene and lycopene in other solvents have not yet been reported A study was undertaken of the variation in the values of $E_{1\,cm}^{1\,per\,cent}$ for the two pigments in various solvents, particularly in CS_2 and petroleum ether which alone can be used in the phase tests Equal fractions of the CS_2 solutions of carotene and of lycopene, were kept overnight in the dark (with a little hydroquinone) in separate flasks and allowed to dry up spontaneously The residues were dissolved in equal volumes of petroleum ether (B P 40°C to 60°C), chloroform, and carbon bisulphide and examined spectrophotometrically The value of the extinction coefficient at the region of maximum absorption, in each case, was determined Since the concentrations of the various specimens of either pigment were identical it followed that the value of $E_{1\,cm}^{1\,per\,cent}$ must be proportional to the observed extinction coefficients Assuming provisionally that $E_{1\,cm}^{1\,per\,cent} = 2,200$ and 2,000 (Gillam, *loc cit*) represent the values for the chloroform solutions of pure carotene and pure lycopene respectively, the corresponding values for CS_2 and petroleum

ether solutions were calculated from the observed data   The results are set out in Table III —

TABLE III

Variations in the values of $E\frac{1\ per\ cent}{1\ cm}$ *for carotene and lycopene in different solvents*

Pigment	Solvent	Refractive index of the solvent	Region of max absorption	Observed ext coef at the region of maximum absorption	Calculated values of $E\frac{1\ per\ cent}{1\ cm}$ in round numbers
Carotene (International standard preparation, 1932)	Petroleum ether (B P 40°C to 60°C)	1 357	4,520	1 26	2,500
	Chloroform	1 449	4,620	1 10	2,200
	Carbon bisulphide	1 628	4,820	0 82	1,600
Lycopene (Prepared as stated above)	Petroleum ether (B P 40°C to 60°C)	1 357	4,740	0 80	2,300
	Chloroform	1 419	4,800	0 70	2,000
	Carbon bisulphide	1 628	5,080	0 50	1,400

Ether and alcohol solutions of the pigments were not studied   According to Kohl (1902) and others, quoted by Palmer (*loc cit*), identical spectra are obtained in solvents of the same refractive indices (ether, petroleum ether and alcohol have similar refractive indices) $E\frac{1\ per\ cent}{1\ cm}$ values for alcohol and ether solutions of carotene and of lycopene will thus be the same as those obtained for the petroleum ether solutions

Absorption photographs were taken with a Hilger's medium-sized quartz spectrograph E316, in conjunction with a rotating sector   The source of light was a spark between tungsten-steel electrodes excited at 15,000 volts   The liquid containers were 1 cm quartz cells   The short wave ultra-violet rays were cut off by filtering them through a thick transparent glass plate

SPECTROGRAPHIC STUDIES OF MIXTURES OF CAROTENE AND LYCOPENE

The absorption spectra of pure carotene and lycopene in carbon-disulphide solutions were first investigated   The observed bands for carotene were at 5,130, 4,820 and 4,520 A U and those for lycopene at 5,480, 5,080 and 4,740 A U   The maximum absorption of the solutions was at 4,820 and 5,080 A U respectively ,

these figures agreed closely with those reported by Gillam and Heilbron (*loc cit*), Schon (*loc cit*) and others It can thus be theoretically expected that in carotene-lycopene mixtures the position of maximum absorption will vary between 4,820 and 5,080 A U, according to the proportions in which each pigment is present

The possibility thereby arises of ascertaining the proportionate composition of carotene-lycopene solutions by means of the position of maximum absorption. The absorption spectra of a few known mixtures of carotene and lycopene in carbon-disulphide solutions were studied Mixtures of carotene and lycopene containing the latter in 20, 40, 60 and 80 per cent proportions showed maximum absorption at about 4,870, 4,920, 4,980 and 5,050 A U respectively figures which agreed roughly with those theoretically expected

It was difficult, with the spectrograph used (Hilger E316), to observe small differences in absorption maxima, and some mixtures appeared to show almost continuous absorption in the region concerned, thus making it difficult to locate correctly the position of maximum absorption It is possible that with an instrument of higher dispersion more accurate readings could be obtained A further complication is that carotene exists in food-stuffs in different isomeric forms (Kuhn and Lederer, 1931, Kuhn and Brockmann, 1933), which show slightly different absorption bands (Morton, *loc cit*) In most common vegetable food-stuffs, however, carotene appears to be present mainly in the β form (Mackinney, 1935, Strain, 1935)

Considering the difficulties of the biological method of assay, and the fact that no simple chemical or physical means have yet been devised for assaying carotene in foods containing both carotene and lycopene, it seems that the method here described may be of some use for rough carotene assays in the case of such foods It is possible that with finer instruments a more accurate technique could be developed The author has used the method for roughly estimating the carotene content of ripe tomatoes and red chillies, which are rich in lycopene The results are given in an accompanying communication (De, 1936)

SUMMARY

1 Carotene and lycopene behaved similarly towards the various reagents and adsorbents used In contra-distinction to their relative solubilities, the former showed greater affinity for adsorption than the latter The difference in their adsorbability establishes the possibility of a chromatographic separation between the two pigments

2 The intensity of absorption of the two carotenoid pigments has been observed to vary from solvent to solvent The values of $E_{1\ cm}^{1\ per\ cent}$ for carotene and for lycopene in chloroform, petroleum ether, and carbon-disulphide solutions have been estimated In the present state of knowledge these figures may be of practical use

3 A rough spectrographic method of estimating carotene in the presence of lycopene is suggested The method seems capable of development, and may prove of use in routine work

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ACTION OF LUGOL'S IODINE SOLUTION ON THE THYROXINIZED HEART

BY

R. K. PAL, D Sc, M R C P (Edin), M Sc, M B (Cal), F R S E,

Formerly Professor of Physiology, Prince of Wales' Medical College, Patna

KALNINS (1928) tried to throw light on the action of thyroxine on the heart and showed that it has no influence on the excitability of the autonomic nerves of the heart of the frog, both the vagus and the sympathetic being unaffected Later on, Priestley, Markowitz and Mann (1931) showed that the rate of hearts of thyroxinized rabbits when perfused and the hearts of thyroxinized dogs when used in the heart-lung preparation was considerably greater than normal, and the tachycardia of experimental hyperthyroidism was not dependent on the central nervous system but on a peripheral mechanism Simultaneously, Yates (1931) also found the same result in rabbits and was of opinion that tachycardia developed in these cases was due to thyroxine acting on all parts of the heart-muscle fibres Experiments in the hands of these workers have proved beyond doubt that thyroxine, whether excessively secreted in the body as in Grave's disease or administered for a considerable period to a healthy animal, acts as a highly toxic substance to the muscle of the heart, leading to tachycardia and various irregularities associated with dilatation of the heart

For some time past Lugol's iodine solution in 10 to 15 minim doses, twice or thrice daily, has come into use as a therapeutic measure in hyperthyroidism to lower down the basal metabolic rate temporarily so that the patient, as a rule, stands the operation of partial thyroidectomy much better Iodine medication is also supposed to prevent the post-operative crisis which otherwise often occurs with fatal results

How iodine acts in these cases is not definitely known Marine (1922), however, considers that the administration of iodine results in the accumulation of colloid material in the alveoli of the thyroid gland leading to increase of pressure in the vesicles, and thereby interfering temporarily with the passage of thyroxine (active secretion) into the blood, which is responsible for the lowering of the basal metabolic rate considerably To ascertain if administration of iodine solution in small amount has any action on the heart under the toxic influence of thyroxine, this investigation was taken up

EXPLANATION OF FIGURES 1 TO 8

FIG 1 Thyroxine in small quantity producing slight increase in the rate of the heart with disappearance of the auricular complex which again reappeared on perfusion with Ringer's solution

,, 2 Lugol's iodine solution making the irregular heart (thyroxinized by repeated subcutaneous injections) normal The height of contraction came down by substituting Ringer for Lugol's iodine solution

,, 3 Lugol's iodine solution making the thyroxinized irregular heart, after direct perfusion of thyroxine solution, normal

,, 4 The irregularity reappeared on stopping Lugol's iodine solution which rendered a thyroxinized heart regular Lugol's iodine when re started caused the irregularity to disappear again

,, 5 Action of Lugol's iodine on a normal heart showing distinct slowing with increased amplitude, which became normal again by replacing the drug with Ringer

,, 6 Potassium iodide solution caused only a temporary improvement on the thyroxinized heart

,, 7 Lugol's iodine solution imparting some amount of regularity to the extremely irregular and slow heart caused by simultaneous perfusion with thyroxine and potassium iodide solution

,, 8 Lugol's iodine solution when perfused along with thyroxine solution was less effective than when perfused alone, in resuscitating a thyroxinized heart

GRAPH

FIG 1

Thyroxin 0·01%, 5.C.C   Ringer

FIG 2

L. Iodine 0·5 p.c.
10.C.C.   Ringer

Lugol's Iodine 0·05%, 2·5 C.C

FIG 3

FIG 4

L. Iodine 0·5 p.c
15.C.C.   L. Iodine Stopped   L. Iodine restarted
0·5 p.c.

FIG 5

L. Iodine 0·05 p.c, 5.C.C   Ringer

Thyroxin 0·005 p.c.   Pot iodide
0·02 p.c.

FIG 6

FIG 7

(Thyroxin 0·005 p.c & Pot iodide 0·02%)   L. Iodine 0·02 p.c
Started

FIG 8

L. Iodine 0·02 p.c
0·005 p.c   Lugol's Iodine
Stopped   L. Iodine 0·02 p.c
Started again
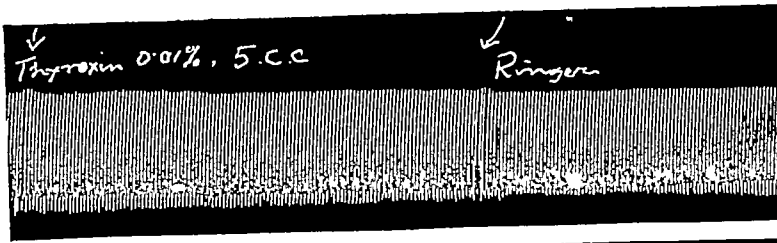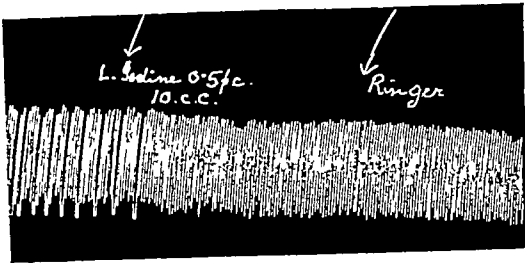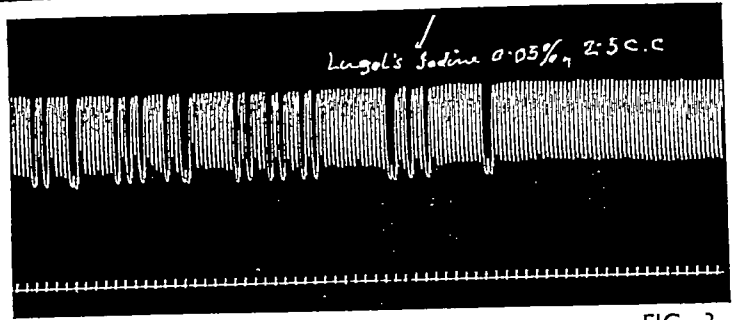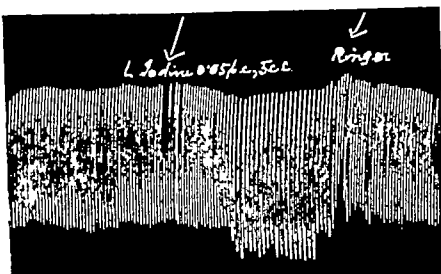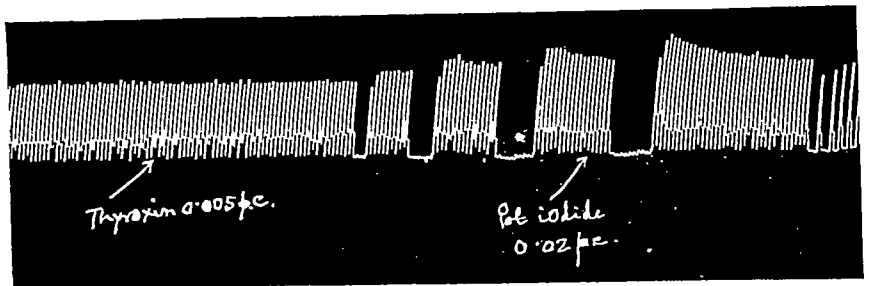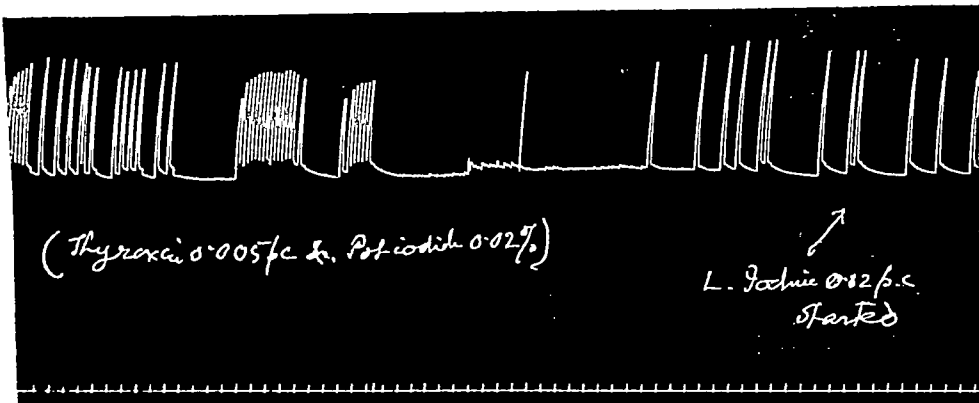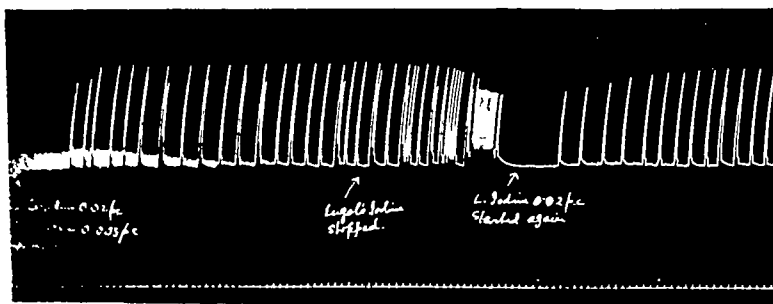
EXPERIMENTAL

A perfusion apparatus containing three bulb funnels with stoppers and stop-cocks, connected to one another by india-rubber tubing (fitted with clips) and Y-tubes, was set up so that they could be opened separately or jointly as required The first funnel contained the simple perfusion fluid which was modified Ringer's solution (Macleod, 1930), the second, a solution of thyroxine in the same solution and the third, Lugol's iodine solution or potassium iodide solution made similarly The level of the fluids in all the funnels was kept the same so that the perfused heart was all throughout subjected to the same fluid pressure The rate of the perfusion flow was also kept fairly constant (35 to 40 drops per minute) throughout all the experiments

Frogs were used for the experiment The heart was perfused through a cannula introduced into the inferior vena cava and the fluid after perfusion was let out through a cut in the aortic arch

Thyroxine used was sodium-thyroxine, B P (Burroughs Wellcome) and the strength of the solution varied from 0 005 to 0 01 per cent Tabloids were well powdered in a mortar and modified Ringer's solution was added slowly up to a certain strength As some insoluble moiety settled down in a few minutes, the solution was centrifuged and the supernatant clear solution was diluted up to the required strength This solution was sometimes injected repeatedly at half an hour's interval into the dorsal lymph sac and sometimes perfused directly into the heart to bring it under the toxic influence of the drug, leading to irregular action of the heart

The perfusion of Lugol's iodine solution diluted with modified Ringer's solution (0 05 to 0 5 per cent) was started later on to note the pharmacological action of the drug on the thyroxinized heart Some normal hearts were also perfused to note the effects, if any, of iodine solution in small doses on the heart Later on potassium iodide solution of the same strength as Lugol's iodine solution (diluted) was also tried alone in order to ascertain if the effects of Lugol's iodine solution on the thyroxinized heart were due to the potassium iodide content of the solution or they are produced by iodine dissolved in potassium iodide solution

The following results are based on about fifty perfusion experiments on frog's hearts thyroxinized previously by repeated subcutaneous injections or by direct perfusion for some time into the heart

RESULTS

Thyroxine solution when perfused for a short time only (5 c c of 0 01 per cent solution) produces slightly increased rate with gradual disappearance of the auricular complex of the contraction, which reappears on perfusion with Ringer's solution (Fig 1)

Repeated injections or direct perfusion for some time (about half an hour) leads to irregularity of the nature of grouped beats Fig 2 shows the appearance of such irregularity after three subcutaneous injections of 1 c c of 0 01 per cent solution of thyroxine at intervals of half an hour The heart was then perfused with Lugol's iodine solution (0 5 per cent, 10 c c) when the irregularity disappeared completely and the heart, to all intents and purposes, became normal Later on

Lugol's iodine solution was replaced by Ringer's solution with the result that the height of contraction came down

Fig 3 shows that perfusion of 2 5 c c of 0 05 per cent solution of Lugol's iodine removed completely the irregularity produced in a heart by direct perfusion with 0 01 per cent solution of thyroxine for about 25 minutes

Fig 4 shows the effect of 15 c c of 0 5 per cent solution of Lugol's iodine in removing the toxic effect of thyroxine, which reappeared on stopping the Lugol's solution   On re-starting the iodine solution the heart again became normal

Fig 5 shows the action of 5 c c of 0 05 per cent Lugol's iodine solution on a normal heart which was slowed down with increased force of contraction   When Lugol's iodine was replaced by Ringer's solution the heart became quicker again with diminished amplitude

Fig 6 shows the effect of 0 02 per cent solution of potassium iodide on a thyroxinized heart   There was slight improvement for a short period only, after which the heart became irregular again

When 0 005 per cent solution of thyroxine solution was perfused along with 0 02 per cent of potassium iodide solution there was no improvement , the heart, on the other hand, became extremely slow   But when 0 02 per cent Lugol's iodine solution was started some regularity was imparted to the extremely slowed heart under the influence of thyroxine and potassium iodide solutions together (Fig 7)

When 0 02 per cent Lugol's iodine and 0 005 per cent thyroxine solutions were perfused together the heart became very slow but fairly regular   On stopping the former, irregularity was noticed but when Lugol's iodine solution was started again the irregularity disappeared and the heart once more began to beat slowly with some degree of regularity (Fig 8)

DISCUSSION

It is not desired to enter into any discussion regarding the nature of the action of thyroxine on the heart, which has been explained by other workers referred to in the beginning of this paper   Experiments in the author's hands showed that thyroxine in small doses leads to an increased rate of the heart with disappearance of the auricular complex   If the toxic action of the hormone continues for some time (as by repeated injections or direct perfusion into the heart) it becomes irregular with grouped beats, this irregularity disappearing completely on administration of Lugol's iodine solution   The same is not without effect on the normal heart as well, and the action manifests itself as slowing in rate with increased amplitude As to which constituent of the Lugol's iodine solution, the solvent or the solute, is responsible for the action on the thyroxinized heart, it has been definitely proved that potassium iodide has only a very temporary effect (if any at all) in removing the toxic action of thyroxine on the heart   If on the other hand, perfusion with Lugol's iodine is carried on along with that of thyroxine solution, the resuscitating action of the former on the heart is less evident

SUMMARY

Lugol's iodine solution lowers the rate but increases the amplitude of contraction of a normal frog's heart

Thyroxine in small doses increases slightly the rate of the heart with diminished auricular complex When the hormone is allowed to act for some time the heart becomes irregular with grouped beats

Lugol's iodine solution can remove completely this toxic effect of thyroxine and make the heart regular The potassium iodide content of the Lugol's solution is not responsible for this action

So Lugol's iodine treatment in hyperthyroidism improves the condition not only by lowering the basal metabolism (the mode of action is obscure) but also by counteracting the toxic effect of the hypersecretion on the heart to a considerable extent

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THE RATE OF ABSORPTION OF GLUCOSE FROM THE GASTRO-INTESTINAL TRACT OF THE CAT AND THE INFLUENCE OF INSULIN ON THE ABSORPTION COEFFICIENT.

BY

H CHAUDHURI

AND

B S KAHALI

(*From the Department of Physiology, Medical College, University of Rangoon, Burma*)

WORKING with non-anæsthetized albino rats, Cori (1925) found that the rate of absorption of glucose from the gastro-intestinal tract was constant and independent of the concentration and the amount of glucose given   Rats from 120 grammes to 180 grammes in weight, starved for 48 hours, were fed concentrated glucose solution by stomach tube   The animals were then killed after a suitable interval, the gastro-intestinal tract was removed, and the amount of reducing substance present in the alimentary tract was determined   The difference between this and the amount of glucose given represented the amount absorbed during that interval of time   The absorption coefficient, that is the amount of glucose absorbed per hour per 100 g body-weight, was 0 178   His findings in general have been confirmed by further works on similar lines by Cori and his associates (1925 to 1929) and also by Auchinachie, Macleod and Magee (1930)   Using the technique of surviving intestine suspended in Tyrode's solution, Auchinachie, Macleod and Magee (*loc cit*) found that the rate of diffusion of glucose into the bath fluid was constant from hour to hour   Macleod, Magee and Purves (1930) found that the optimum concentration was 0 75 molar glucose solution   Using a modified Cori method, Magee and Reid (1931) in unanæsthetized rats, anæsthetized cats and rabbits found that the absorption coefficients were 0 167, 0 021 and 0 033 respectively with 0 75 molar glucose   Their cats and rats were fed by glucose solution by the stomach tube, while the rabbits received their glucose with injection into the duodenum   It was also found that the absorption coefficients increased with increasing concentration of glucose solution fed up to 0 75 molar, but diminished with further increase of concentration   Percentage increase and decrease of the absorption coefficient with varying concentration of glucose, though small, was found statistically to be

significant Trimble, Carey and Maddock (1933) using Cori's method found the absorption coefficient to be 0 099 in dogs

But Pierce, Osgood and Polansky (1929) using the same method found that the rate of absorption depended on the amount of glucose present in the intestines and consequently fell off during the second and subsequent hours Cori *et al* (1929) pointed out that this was due to the insufficient amount of glucose fed to continue absorption at the original level Burget, Moore and Lloyd (1932) and Mackay and Bergman (1933) using Cori's method with a feed of sufficient glucose reported similar falling off of the absorption coefficient with time The latter workers also found that the absorption coefficient varied significantly with the concentration of glucose solution fed This brief résumé shows that unanimity of opinion about this important matter has not yet been reached All workers in this field agree that absorption of glucose is a vital process but the rate at which it is absorbed and the factors which influence it are as yet not clearly understood The experiments in the present work have been carried out in two groups A and B to determine —

A   The absorption coefficient of glucose from the intestinal tract of amyta-
       lized cats
B   The influence of insulin on such absorption, if any

Group A   The absorption of glucose from the intestinal
tract of amytalized cats

Methods

Cats between 1 5 kg to 3 kg weight were used They were kept under observation for a few days and fed with milk, fish and sufficient rice They were fasted for 24 hours before being used for the experiment Longer fasting reduces the rate of absorption of glucose (Cori and Cori, 1927) Water was allowed *ad libitum* The cats were anæsthetized with amytal about 0 09 g per kg intraperitoneally Anæsthesia was complete within fifteen to thirty minutes After anæsthesia was complete the abdomen was opened by a median incision from the xiphoid process to the symphysis pubis Ligatures were applied at the duodeno-pyloric and the ileocæcal junctions The superior mesenteric vein was exposed in its bed for collection of blood The cut ends of the abdominal parietes were apposed with clips and all exposed surface covered with lint moist with warm normal saline One hour after this operation 0 2 c c to 0 3 c c of blood was taken from the superior mesenteric vein in an oxalated tube The glucose solution at room temperature (28°C to 31 4°C) was then introduced into the duodenum with a fine needle and a calibrated Record syringe The concentrations of the glucose solution used were 5 g , 10 g and 20 g per cent Samples of blood were taken periodically for blood sugar estimation After a suitable interval, usually between 1 and 2 hours, the mesenteric vessels were tied and the small intestine was removed The outside of the intestine was washed rapidly under tap water The whole procedure from ligaturing the mesenteric vessels to the washing of the outside of the intestines did not take more than one minute The intestine was then opened along the mesenteric border, the contents were collected in a beaker The opened intestine was then thoroughly washed with distilled water, usually about 300 c c , until the last washings were free from reducing substance The contents and washings were mixed and the amount of reducing substance in it was estimated The amount of glucose absorbed was found by

subtracting the amount present in the intestines from the quantity given The contents of the large intestine were examined in each case for the presence of reducing substance Except in one case (not included in the result) the large intestine did not contain any glucose

Analytical procedure

Benedict's qualitative reagent was used to detect the presence of reducing substance The estimations of reducing substance (glucose) in the protein-free gastric and intestinal contents were done by the Benedict's method (Peter and van Slyke, 1932) The proteins were precipitated by heating the washings to the boiling point and then acidifying with a few drops of 10 per cent acetic acid A few drops of 10 per cent calcium chloride were also added to complete the precipitation Blood sugar was estimated by the method of Folin (1926) and by the method of Hagedorn and Jensen (Cole, 1933)

Control experiments

1 *The dose of amytal required to anæsthetize cats* —Amytal (Eli Lilly & Co , Ltd , New York) was used as an anæsthetic as it has been shown by Page (1923), Mulinos (1928) and Deuel *et al* (1926) and others not to cause any disturbance in the carbohydrate metabolism The dose of 0 05 g per kg , as recommended by the manufacturer, was found to be inadequate to produce complete anæsthesia 0 09 g per kg always produced anæsthesia within 15 minutes The condition of the animals remained satisfactory for 3 or 4 hours as judged by respiration and pulse rate

2 *Reducing substance in the stomach and intestines of fasted animals* —In ten control experiments no reducing substance was found by the Benedict's method either in the stomach or in the small intestine of the cat fasted for 24 hours

3 *Effect of anæsthetic and operation* —The blood sugar tends to rise for one hour after an operation under anæsthesia Within one hour it attains its maximum and remains almost at that level for one or two hours, as the protocol of one of the typical experiments will show

25th August, 1933 Male cat, 2 5 kg

Amytal, 2 5 g at 11 a m

Operation completed by 11-30 a m

Blood sugar at	11-32 a m	0 117	per cent by Folin method
,,	12 noon	0 131	,,
,,	12-30 p m	0 188	,,
,,	1 p m	0 188	,,

Evans *et al* (1931) have also reported a gradual but small increase of blood sugar for the later hours after amytal

Results

Experiments with 10 per cent glucose solution given intraduodenally

The amount of glucose injected into the duodenum was approximately 1 g per kg body-weight The duration of absorption varied from 90 to 105 minutes

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The results are recorded in Table I   The average absorption coefficient was 0 0486

It was further observed that the average fasting blood sugar of anæsthetized and operated cats, one hour after operation, was 0 113 per cent   The average blood sugar, one hour after intraduodenal glucose (1 g per kg body-weight given in 10 per cent solution) was 0 236 per cent, a rise of about 108 per cent over the fasting level

The protocol of one of the typical experiments is given below —

7th December, 1933   Experiment No 2

      Male cat 3 65 kg

      10-30 a m   3 6 c c of 10 per cent amytal intraperitoneally

      11-5 a m   Operation was completed

      12-20 p m   First sample of blood taken glucose 3 348 g in 10 per cent solution intraduodenally

The experiment terminated at 2-5 p m

    12-30 p m   Blood sugar 0 113 per cent

    12-50 p m   0 171

    1-23 p m   0 214   ,

    1-50 p m   0 225

TABLE I

Rate of absorption of glucose of 10 per cent strength from the small intestine of cats

Experiment number	Sex	Weight in kg	Absorption period in minutes	Glucose given in g	Glucose recovered in g	Glucose absorbed in g	Glucose absorbed in g per 100 g body weight per hour
1	♂	2 27	90	2 048	0 564	1 484	0 049
*2	♂	3 65	105	3 348	1 268	2 080	0 0321
17	♂	2 03	95	1 888	0 324	1 564	0 0486
18	♂	2 74	90	2 511	0 525	1 986	0 0483

   * There was considerable fecal matter in the small intestine   This has not been taken into account in calculating the average

Experiment with 20 per cent glucose solution —The amount of glucose given was approximately 2 g per kg   The duration of the experiment varied from 90 to 130 minutes   The results are recorded in Table II   The average absorption coefficient was 0 038 for the entire group or 0 034 for the male cats only

TABLE II

The rate of absorption of glucose of 20 per cent strength from the small intestine of cats

Experiment number	Sex	Weight in kg	Absorption period in minutes	Glucose given in g	Glucose recovered in g	Glucose absorbed in g	Glucose absorbed in g per 100 g body weight per hour
21	♂	3 00	95	5 580	4 116	1 464	0 0308
22	♂	2 50	130	4 650	2 553	2 097	0 0387
23	♀	2 10	90	3 906	2 300	1 606	0 0506
42	♂	1 85	70	3 348	2 651	0 697	0 0320

Experiment with 5 per cent glucose —The amount of glucose injected was about 1 g per kg body-weight   The duration of absorption varied from 60 to 100 minutes   The results are recorded in Table III   The average absorption coefficient was 0 031 for the entire group

TABLE III

The rate of absorption of glucose of 5 per cent strength from the small intestine of cats

Experiment number	Sex	Weight in kg	Absorption period in minutes	Glucose given in g	Glucose recovered in g	Glucose absorbed in g	Glucose absorbed in g per 100 g body weight per hour
24	♀	1 2	100	1 116	0 357	0 759	0 038
25	♀	1 48	68	0 651	*Nil*	0 651	0 038
39	♂	2 27	60	1 628	1 034	0 594	0 026*
40	♀	2 65	77	2 290	1 299	0 991	0 024
41	♀	2 04	60	1 660	1 064	0 596	0 029

* There were many worms in the intestines

GROUP B   EFFECT OF INSULIN ON THE ABSORPTION OF GLUCOSE FROM THE SMALL INTESTINES

Methods

The experiments were done exactly in the same way as the previous groups of experiments but the animals in these experiments received a dose of insulin either 1 unit per kg of body-weight subcutaneously (group A) or 0 5 unit per kg intravenously (group B) at the time the 10 per cent glucose solution was injected into the small intestine

The results are recorded in Table IV, *A* and *B*,

The average absorption coefficient in group A is 0 041 and that in group B 0 0396   The average absorption coefficient of both groups is 0 04

It was further observed that the average post-operative fasting blood sugar of these animals was 0 117 per cent and the average blood sugar, one hour after intraduodenal glucose (1 g per kg body-weight in 10 per cent solution) with simultaneous injection of insulin (1 to 0 5 unit per kg body-weight) was 0 131 per cent, a rise of 12 per cent over the fasting level

The protocol of one of the typical experiments is given below —

    1st February, 1934   Experiment No 13

        Female black cat 2 47 kg

        9-45 a m Amytal 2 4 c c of 10 per cent solution intraperitoneally

        10-10 a m Operation was completed

        11-20 a m First sample of blood taken glucose 2 297 g in 10 per cent solution intraduodenally

        11-25 a m Insulin 2 5 units subcutaneously   Blood was taken at half hourly intervals

The experiment was terminated at 12-50 p m

Fasting blood sugar 0 115 per cent

½ hour after glucose blood sugar—0 156 per cent

1 hour after glucose, blood sugar—0 170 per cent

TABLE IV

Effect of insulin on the rate of absorption of 10 per cent glucose from the small intestine of cats

A

Experiment number	Sex	Weight in 1 g	Absorption period in minutes	Glucose given in g	Glucose recovered in g	Glucose absorbed in g	Glucose absorbed in g per 100 g body-weight per hour
13	♀	2 47	90	2 297	0 753	1 544	0 0415
14	♂	2 00	90	1 860	0 624	1 236	0 0412
15	♂	1 85	100	1 720	0 427	1 293	0 0419
16	♂	3 07	95	2 790	1 400	1 390	0 0286

B

Experiment number	Sex	Weight in 1 g	Absorption period in minutes	Glucose given in g	Glucose recovered in g	Glucose absorbed in g	Glucose absorbed in g per 100 g body-weight per hour
29	♂	1 83	90	1 700	0 486	1 214	0 0441
30	♀	1 13	93	1 302	0 510	0 792	0 0352

Control experiments with insulin but without intestinal glucose were performed to find the effective doses of insulin given either subcutaneously or intravenously It was found that both 0 5 unit and 1 unit dose per kg subcutaneously reduced blood sugar to the same extent Blood sugar tended to fall even after 90 minutes Percentage fall in the case of 1 unit dose was slightly higher than the percentage fall in the case of 0 5 unit dose

Intravenously insulin lowered blood sugar within 6 minutes in 1 unit doses but with both doses the percentage fall attained its maximum within half to one hour Subsequently the blood sugar tended to rise

Results are recorded in Table V —

TABLE V

Effect of insulin given subcutaneously or intravenously on blood sugar of cats

Experiment number	Sex	Units of insulin per kg		Initial blood sugar	PERCENTAGE FALL OF BLOOD SUGAR AT INTERVALS					
					6 to 10 mts	20 to 30 mts	40 to 50 mts	60 to 65 mts	80 to 90 mts	120 mts
10	♀	1	subcutaneously	0 100		0		50	67	
11	♀	0 5	,,	0 105		14 2		45 7	61	
12	♀	1	,	0 115		7		40	63	88
27	♀	1	intravenously	0 076	2 6	50		47		
29	♂	0 5	,,	0 102		25	45	50	42	

DISCUSSION

Macleod *et al* (*loc cit*) found that the rate of transfer of glucose from the intestines was maximum with a 0 75 molar solution of glucose Magee and Reid (*loc cit*) later on reported that this was also the optimum concentration of glucose for absorption from the gastro-intestinal tract of rats, cats and rabbits The solutions of glucose used by them in different species of animals were 0 5, 0 75, 1, 2 and 3 molar Results of the present work also suggest that there is an optimum concentration for the absorption of glucose The concentrations of glucose used were 0 27 molar (5 per cent), 0 55 molar (10 per cent) and 1 1 molar (20 per cent) and the absorption coefficients were 0 031, 0 0486 and 0 034 respectively Apparently the optimum concentration for absorption of glucose lies between 0 55 molar (as in these present experiments) and 0 75 molar (as found by Magee and Reid)

Cori and Cori (1928) reported that insulin even in very large doses had hardly any effect on the absorption coefficient of glucose in rats   Injecting 15 units of insulin per 100 g of body-weight, they found that the average absorption coefficient was only 5 9 per cent more than the absorption coefficient of glucose in the rats without insulin   The result of the present work shows that insulin injected in 1 to 0 5 unit per kg of body-weight does lower the absorption coefficient from an average of 0 042 to 0 040, i e , about 16 6 per cent   This dose of insulin (1 to 0 5 unit per kg body-weight) produced a 50 per cent fall of blood sugar in amytalized and operated cats in one hour   The same dose of insulin injected into the anæsthetized operated cats into the duodenum of which animals glucose (1 g per kg body-weight in 10 per cent solution) was simultaneously introduced did not produce any fall of blood sugar in one hour   The average blood sugar, in these cases, at the end of one hour, was 12 per cent over the pre-insulin fasting level   The post-glucose rise of blood sugar in anæsthetized cats, which did not receive insulin, was 108 per cent over the fasting level, one hour after intraduodenal glucose (1 g per kg body-weight in 10 per cent solution)   This shows that insulin in this dose had its usual action on the carbohydrate metabolism of the cats which received glucose intraduodenally at the same time   During such an action of insulin, the absorption coefficient of glucose is slightly lowered   The average absorption period of Cori's animals was 240 minutes, while the average absorption period in the work reported here was 98 minutes   Whether the longer absorption period and the much higher dose of insulin (about 150 to 300 times more) used would explain the discrepancy between their results and our findings is a problem for further investigation Moreover, the average blood sugar of the glucose-fed rats without insulin in Cori and Cori's experiments was 158 mg per cent and that of glucose-fed rats with insulin was 72 mg per cent which is less than the usual fasting glucose content of blood In the experiments reported here in insulin-injected cats receiving glucose intraduodenally blood sugar did not fall below the fasting level but was always higher than the fasting level   Perhaps a lowering of blood sugar *per se* may influence the rate of absorption of glucose

A difference in some of the aspects of the metabolism of carbohydrates between two sexes has been found to exist in rats (Butts and Deuel, 1933)   In some of our experiments, too a slight difference in the absorption coefficients of the two sexes was found but the difference was always too small to invalidate the average results Moreover, our experiments do not include sufficient animals of both sexes to form any general conclusion

Summary

(1) There is an optimum concentration for the absorption of glucose from the gastro-intestinal tract of cat   this lies between 0 55 molar and 0 75 molar

(2) The average absorption coefficient of glucose with 0 55 molar solution injected directly into the small intestine is 0 048

(3) Insulin injected simultaneously with glucose lowers the absorption coefficient

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HÆMATOLOGICAL STUDIES IN INDIANS

Part V

RED BLOOD CELL MEASUREMENTS

BY

L EVERARD NAPIER, M R C P (Lond),

AND

C R DAS GUPTA, M B (Cal), D T M (Bengal),
Calcutta School of Tropical Medicine

(*An Inquiry under the Endowment Fund of the School*)

HISTORICAL

In hæmatological investigations, it is almost as essential to know the size of the red blood corpuscles as it is their number and their total hæmoglobin content

Curiosity regarding the size of the red blood cell dates from the days of van Leeuwenhock, and more than two hundred years ago Dr James Jurin attempted to give exact figures for the diameters of red blood cells , his methods were, naturally, crude and his results would not have been of much value to the modern hæmatologist , in his first attempt he arrived at a figure that corresponds to 7 75μ, but in a subsequent attempt he was a little less successful with a figure corresponding to 12 5μ During the next hundred and fifty years or so, many more attempts were made with instruments of increasing precision , most of the figures given were still well wide of the mark   Estimations were always based on a small number of observations , a paper that appeared in the *Indian Medical Gazette* in 1883 gave the diameter of the human erythrocyte based on the measurements of 10 cells, as 7 3μ to 7 9μ

Malassez (1889) appears to be the first to have attached any significance to the variations in the size of the individual cells in any one person's blood and to have worked out the mean diameters on a reasonable number of observations

To come down to the present generation of workers, Price-Jones's (1910) was the voice in the wilderness that prepared the way for the coming of the new hæmatology of the present decade, by his technique individual cells are measured and a frequency curve drawn, so that not only the mean diameter but also the dispersion around that mean can be calculated. Then in 1924 Pijper revived the diffraction method of measuring the diameters of small objects and applied it to red blood cells, various techniques were elaborated for putting this method on a clinical basis and Eve (1928) devised a cheap and simple halometer which has been used extensively in this country. It has been claimed that the mean diameter of the cells can be estimated with considerable precision and that some idea of the dispersion can also be obtained by the diffraction method (Pijper 1935).

The hæmatocrit was first introduced in the early part of this century, apparently as an instrument for obviating the tedium of counting the red cells, but fell into disfavour because it was found that the correlation factor between the volume of packed cells and their number was not a constant one, or, in other words for the very reason that it is now coming into use again as a means of estimating cell volume. More satisfactory estimations of cell volume are, however, obtained by using tubes of wider bore and larger quantities of blood. By this method only the mean cell volume is obtained and there is no indication of the dispersion around the mean.

Halometric Readings

The theory on which this method is based is perfectly sound and has been generally accepted. Further the method has been used extensively by British workers and the accuracy of the findings do not appear to have been questioned. Maplestone measured the diameters of 500 erythrocytes of each of ten normal Indians by Price-Jones's method and found the mean diameters to correspond very closely with the halometric readings on the blood films from the same individuals, his work was examined statistically by Chaudhuri (1933) who agreed that the correlation was a good one. In view of these facts we felt justified in accepting the method as a means of finding out, at least roughly, the mean diameters of the red cells of the normal and anæmic persons that were included in the investigations reported in this series of papers. Whilst normal individuals were being examined the readings were not sufficiently removed from the normal to make us doubt their accuracy, but immediately we used the method on bloods of low hæmoglobin content we found considerable difficulty in making readings and when we made them they were frequently anomalous, that is to say, cells of large diameter were often associated with extreme hypochromia, and vice versa. In our earlier papers we have reported the halometric readings, but with some misgivings which we expressed.

We took an early opportunity of checking some of our readings by Price-Jones's method in anæmic patients in the way that Maplestone did with normal Indians. For this, samples were taken from our anæmic series amongst Assam tea-garden coolies, cases were picked at random from the patients that we were investigating, except that we deliberately included two that showed slight hyperchromia and that were therefore in all probability also slightly macrocytic, this in fact they turned out to be. The routine halometric readings were made immediately the film had dried but the Price-Jones's curve was drawn from the measurements made in Calcutta from the same blood slides some months later.

The figures obtained were as follows —

TABLE I

Serial number	Mean diameter of about 500 cells, obtained by Price Jones's method	Mean of three readings, obtained by using Eve's halometer
	u	*μ*
1	7 72	7 81
2	7 71	7 70
3	7 17	7 48
4	7 13	7 59
5	6 93	7 64
6	6 92	7 27
7	6 91	7 67
8	6 21	7 75
9	5 96	7 56
10	5 73	7 32
AVERAGES	6 84	7 58

The coefficient of correlation, worked out according to the formula

$$ r = \frac{\leq (x - \bar{x})(y - \bar{y})}{\sqrt{\leq (x - \bar{x})^r \leq (y - \bar{y})}} \text{, is 0 4106} $$

This figure is very low and indicates that one or other method (or both methods) is inaccurate, but it is much higher than one would have expected from a casual perusal of the series, and does not give a fair indication of the practical value of the halometric readings   If we look at the figures from a practical point of view, we see that in the Price-Jones's series three cases are slightly but definitely microcytic and three are markedly microcytic, whereas the halometric readings do not indicate that any of these six cases are microcytic   in fact three give a reading higher than the mean of the series   The fallacies of the Price-Jones's method we shall discuss below, but if allowance were made for the maximum error possible by the Price-Jones's method, the coefficient of correlation would still be a small one   We must therefore assume that, as far as this series was concerned, the halometric readings were of little value

Discussion —We fully realize that by reporting our failure to obtain satisfactory results with Eve's halometer we are laying ourselves open to obvious criticism we can already hear a proverb about workmen and their tools and the remark that no method is entirely fool-proof being used against us   We can only take a humble attitude and say that our conclusion is the result of an honest attempt by two not inexperienced workers to make use of a method that has the great advantage of being a simple one rapidly carried out

However, we cannot help feeling that we are not alone in our failure to obtain accurate results with this instrument   For example, we notice that American workers seldom use the method and many of their most recent books on laboratory

technique make no reference to it In India, the method seems to have found favour amongst clinicians as a rapid method of differentiating between macrocytic and microcytic anæmia, but we firmly believe that it has been responsible for much mistaken diagnosis as results are seldom checked by other methods Further, Mudaliar and Rao (1932) used the instrument in an investigation into anæmia of pregnancy with results that support our contentions they give a halometric reading of 4 3 (which corresponds to 7 98μ) for 12 out of 20 cases in which they used the halometer, in these twelve cases the colour indices varied from 1 5 to 0 6 The coefficient of correlation between the colour index and the halometric reading in the 20 cases was + 0 179 There should be a much closer correlation between the colour index and the size of the red cells in this condition

In another series also reported in this *Journal* (Hughes and Shrivastava, 1931), there was even less correlation between the colour indices and the halometric readings, in only 1 out of 13 cases of splenomegaly was the colour index above 1 whereas the mean diameter of the series, according to the halometric reading was 7 77μ, the smallest mean diameter being 7 32μ That is, in 13 out of 14 cases there was hypochromia associated with normal-sized or macrocytic red cells, a most unusual finding The correlation coefficient between the colour indices and the cell diameters was 0 039

Wherever lay the fault, we felt that our inability to manage the halometer justified us in turning to other methods for estimating the size of red cells

PRICE-JONES'S METHOD

Price-Jones (1933) in his book does not give any elaborate details of technique, probably for the reason that it is better for each worker to work out the details of procedure according to his special circumstances Thus we did, and in doing so encountered certain difficulties that are perhaps worth recording

The films were made from blood taken directly from the finger on slides that had first been cleaned by the ordinary routine laboratory procedure with potassium bichromate and had subsequently been polished with jewellers' rouge They were dried rapidly in the air and stained with Leishman's stain

For projection and drawing an Edinger projection apparatus was used First of all it was necessary to 'set' the apparatus to obtain a magnification of exactly one thousand times, this was done by drawing a scale—a series of 16 short parallel lines with 1 centimetre interval between each – on a piece of Bristol board placing a stage micrometer over the oil immersion lens, focusing this on to the Bristol board, and adjusting the apparatus until the lines on the Bristol board correspond with the projected scale of the stage micrometer (one cubic centimetre corresponding to 10μ) Here the first difficulty arises, if the lines correspond in the centre of the field, they will not do so at the periphery and vice versa We adjusted the apparatus in such a way that the whole length of 15 centimetres corresponded with 150μ of the stage micrometer

The place of the stage micrometer is then taken by the blood film to be examined A field containing a large number of discrete red cells is chosen, the focus adjusted so that the outline of each cell is sharp, and these outlines are then traced with a sharp drawing pencil, a fresh area of Bristol board is moved under the circle of light, the slide moved, a second field is focused on the

Bristol board, and the cell outlines again traced the procedure is repeated until at least 500 cells have been drawn

The longest and the shortest diameters of each cell are measured and the mean of the two diameters entered on to the card The easiest way of measuring the diameters is by means of a transparent diagonal scale (Text-fig 1) With this it is
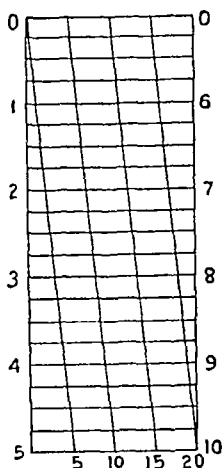
TEXT FIG 1

Diagonal scale for measuring
red cell diameters (Reduced
to half original size)

possible to measure to the nearest 0 25 millimetre ($=0 25\mu$) with considerable precision The mean diameter of each cell outline is thus recorded in multiples of 0 125 of a millimetre (In practice it is only necessary to write the first place of decimals, e g , 7 1 for 7 125 and 6 3 for 6 375, if one remembers subsequently that the shorter figure is only a symbol) If the size of the cell is written on the card against each cell outline, it is possible for another worker to check the measurements this we did frequently until we found that there was seldom any disparity between the measurements of the two observers

We then tabulated the measurements field by field until it was seen that the next field would take the number beyond 500 , when this stage was reached the next field was divided into segments, of quarters or even eighths (*see* Text-fig 2), and the cells were counted a segment at a time until 500, or just over 500, cells had been counted

It was observed that the cells in the centre of the circle tended to be smaller than those at the periphery In order to ascertain the magnitude of this difference in the size of the cells in different parts of the field, three circles were drawn, one of 2 5 cm radius, one of 5 cm radius, and one of 7 5 cm radius, so that the fields were divided into three areas , the cell sizes were tabulated according to the area in which they occurred as well as according to fields Finally, a combined frequency table was constructed by summing the frequency tables of all the fields from one case

Thus, for each case we had a full frequency table of the 500 cells, three frequency tables numbered I, II and III, for cells inside the inner circle, for those between the inner and middle circles and for those between the middle and outer circles respectively, and five or more (12 in one case) frequency tables for the separate fields, denoted as A, B, C etc. In addition three combined frequency tables in
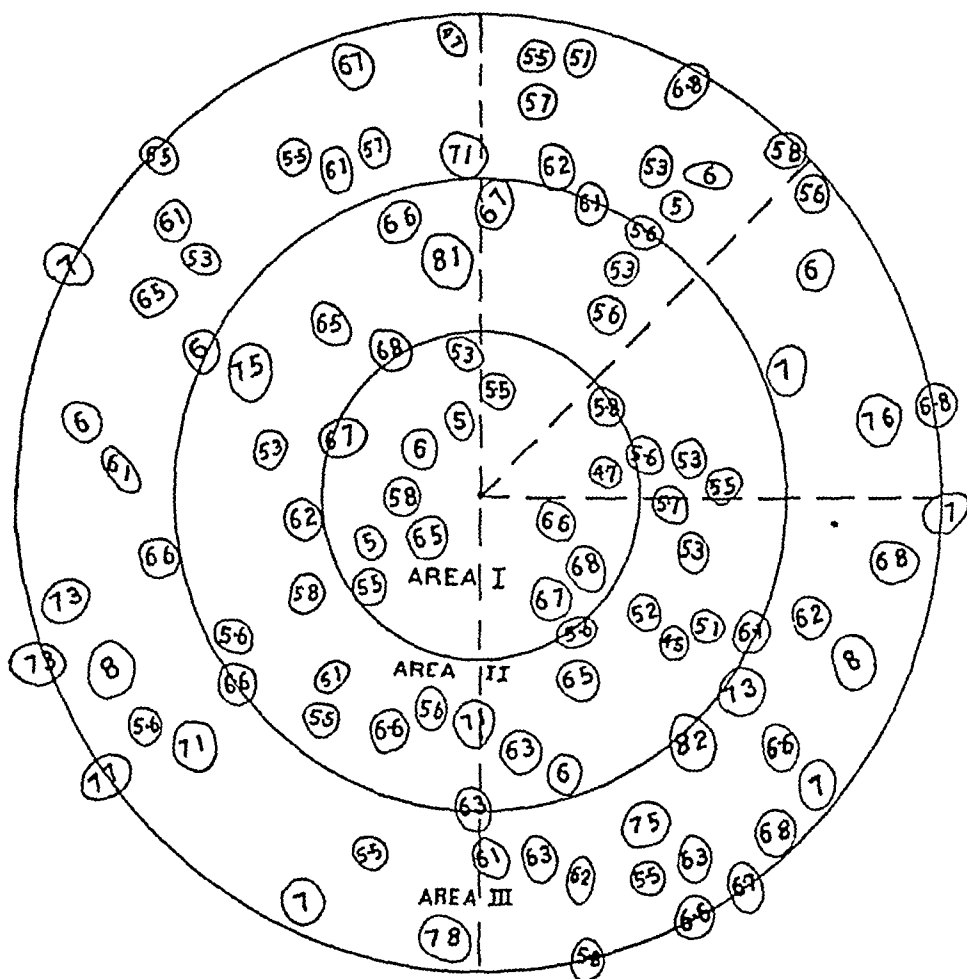
TEXT FIG. 2.

A projected field with cell outlines traced and the mean length of the diameters written in each. The field is divided into three areas by means of three circles, and into segments for facilitating the counting of approximately the correct number of cells. (Reduced to about three quarters original size.)

which all the cells from all* the cases grouped according to the areas in which they occurred, I, II or III, were prepared.

The mean, the standard deviation, the standard error, and the square of the standard error for each of these groups of cells was calculated (*see* Protocols I and II).

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* Nine only are included as in one case the magnification was not exactly 1 000 times and the correction could be applied only to the final figures.

PROTOCOL I

Case D 4

	Mean	Standard deviation 's'	Number of cells 'n'	Standard error $\frac{s}{\sqrt{n}}$	Standard error squared $\left(\frac{s}{\sqrt{n}}\right)^2$	Deviation of mean from mean of population 'd'	Fisher's 't' $\frac{d}{\frac{s}{\sqrt{n}}}$	Range of probability 'P'
Total	7 72	± 0 7640	506	0 0340	0 00115600			
Area I	7 58	± 0 7250	55	0 0978	0 00956484			
„ II	7 51	± 0 7560	177	0 0568	0 00322624			
„ III	7 98	± 0 6920	274	0 0418	0 00174724			
Field A	7 31	± 0 8456	45	0 12605	0 01588965	0 21	1 667	0 1 to 0 05
„ B	7 87	± 0 7802	35	0 1319	0 01739761	0 15	1 136	0 3 to 0 20
„ C	7 55	± 0 6670	39	0 1068	0 01140624	0 17	1 589	0 2 to 0 10
„ D	7 67	± 0 7687	37	0 1264	0 01597696	0 05	0 397	0 5 +
„ E	7 71	± 0 6808	43	0 1038	0 01077444	0 01	0 096	0 5 +
„ F	7 62	± 0 8230	47	0 1200	0 01440000	0 10	0 850	0 4 to 0 30
„ G	7 69	± 0 7291	41	0 1139	0 01297321	0 03	0 263	0 5 +
„ H	7 83	± 0 4207	41	0 0657	0 00431649	0 11	1 667	0 1 to 0 05
„ I	7 76	± 0 7075	43	0 1079	0 01164241	0 04	0 371	0 5 +
„ J	7 84	± 0 7850	41	0 1226	0 01503076	0 12	0 976	0 4 to 0 30
„ K	7 67	± 0 6220	42	0 0960	0 00921600	0 05	0 521	0 5 +
„ L	7 80	± 0 9140	52	0 1267	0 01605289	0 08	0 630	0 5 +

PROTOCOL II

Case 42

	Mean	Standard deviation 's'	Number of cells 'n'	Standard error $\frac{s}{\sqrt{n}}$	Standard error squared $\left(\frac{s}{\sqrt{n}}\right)^2$	Deviation of mean from mean of population 'd'	Fisher's 't' $\frac{d}{\frac{s}{\sqrt{n}}}$	Range of probability 'P'
Total	5.99	± 0.8909	300	0.0898	0.00158404			
Area I	5.74	± 0.8573	64	0.1072	0.0119184			
„ II	5.86	± 0.8800	160	0.0696	0.0048441			
„ III	6.14	± 0.8562	276	0.0515	0.00265225			
Field A	6.10	± 0.9587	106	0.0932	0.00868624	0.11	1.180	0.3 to 0.20
„ B	6.04	± 0.9106	124	0.0818	0.00669124	0.05	0.611	0.5 +
„ C	6.02	± 0.7481	98	0.0797	0.00635209	0.03	0.376	0.5 +
„ D	5.79	± 0.8439	110	0.0804	0.00646410	0.20	2.488	0.02 to 0.01
„ E	5.98	± 0.9274	72	0.1093	0.01194649	0.03	0.275	0.5 +

Variations in the diameter of the cell outline according to the distance from the centre of the field

Table II shows the mean, standard deviations, etc , of all* cells, irrespective of the cases to which they belong, in each of the different areas, I, II and III   It will be seen that the mean size of the cells increases from within outwards, and that the differences between any two are significant, the difference between I and II being 2 62 times the standard error of this difference, between I and III 7 52 times, and between II and III 7 54 times

TABLE II

Areas	I	II	III
Number of cells	504	1 545	2,454
Mean diameter	6 59μ	6 73μ	6 97μ
Standard deviation	±1 045	±1 020	±1 126
Standard error	±0 0466	±0 0260	±0 0227
(Standard error)²	0 002172	0 000676	0 000515

It is thus apparent that the difference between the mean diameters of the cells in the different circles is a real one   If the cases are considered separately (Table III), it will be seen that though the differences are fairly constant, they are not always statistically significant , this is what one would expect where smaller numbers are involved

TABLE III

Showing the mean diameters of the cells falling in different areas in the field and the significance of the difference between different pairs

Cases	MEAN DIAMETER IN DIFFERENT AREAS, IN MICRONS			SIGNIFICANCE OF DIFFERENCES		
	I	II	III	I and II	I and III	II and III
D 4	7 58	7 51	7 88	—	s	S
35	6 81	7 09	7 35	s	S	S
32	6 18	6 05	6 31	—	O	S
42	5 74	5 86	6 14	O	S	S
B	7 36	7 53	7 89	O	S	S
43	5 62	5 76	5 74	O	O	—
29	6 79	7 04	7 45	O	S	s
33	6 56	6 83	7 10	s	S	S
55	6 82	6 80	6 95	—	O	s

— = Negative difference
O = Positive difference but not ' significant '
s = Difference more than twice standard error, i e , ' significant '
S =   ,,   ,,   three times standard error, i e , ' very significant '

* Nine only are included as in one case the magnification was not exactly 1,000 times and the correction could be applied only to the final figures

10

This difference in the size of the magnification at the periphery and at the centre of the field is what one would expect, but it does not seem to have been taken into consideration by other workers. The distance of the drawing paper from the lens of the eye-piece (No 4) was almost exactly 25 cm, measured vertically, by a simple geometrical calculation we decided that the image at the periphery of the field would be $\sqrt{\dfrac{25 + (7\,5)}{25°}}$ or 1 044 times the size of the image at the centre

That is to say, if a cell was 7 0 millimetres at the centre it would be $7\,0 \times 1\,044$ or 7 308 millimetres at the periphery. Now the actual mean difference in the sizes of the cells in area I and area III are greater than 0 308, whereas it should be less, as we were not measuring only the cells at the centre and periphery, respectively, but all the cells in the two areas. It is apparent therefore that the calculation we made was not correct, and on further consideration—and after consultation with our colleagues (*vide* note by Dr Raja)—we realized that to calculate the exact figure a knowledge of optics greater than we possessed was necessary

The eye-piece micrometer scale was made to correspond with the drawn scale on the Bristol board between points at the periphery of the field, or as near to it as a sharp focus could be obtained, if a circle is drawn to divide the whole field into two equal parts, it will cut the 7 5 cm radius of the field at a point 5 3 cm from the centre, and it is also at this point that the magnification is exactly correct, that is midway between the magnification at the periphery and at the centre, thus there will be as many cells—all slightly too large—outside this dividing circle as there will be inside it, the latter being all slightly too small, and the mean size of the cells of the whole area will be unaltered, but the dispersion will be slightly greater than it should be

In this series of measurements no serious error has to be corrected but the fact that there is a difference between the magnification at the centre and at the periphery is not emphasized by writers on the subject of cell measurements and, if the magnification is adjusted to be correct at the centre then cells in the outer half of the fields should not be included, or, conversely, if the magnification is adjusted to be correct over the whole diameter of the field, all the cells in the field and not merely those in centre must be measured

Variations in the size of the red cells in different parts of the film

In order to complete the 500 cells it was necessary to take in each blood film from 5 to 12 fields, according to the nature of the film. In normal films we have found that no difficulty is experienced in including an average of 100 discrete cells, but in films from anæmic individuals it is often difficult to find fields with as many as even 50 cells in them. In this series there were very few fields containing less than 40 cells, the largest field contained 124, and the average was about 70 cells per field

In four cases, the mean sizes of the cells in the different fields in the same film fell within a range of $0\,36\mu$, that is to say, if the mean size of the cells in one field was as high as $7\,27\mu$, in no instance in the same film was the smallest mean less than $6\,91\mu$. In the other six cases the range was $0\,60\mu$, $0\,76\mu$, $0\,80\mu$, $0\,81\mu$, $0\,95\mu$ and $1\,36\mu$, in this last instance the largest mean was $8\,46\mu$ and the smallest $7\,10\mu$

We adopted two methods to test the significance of the differences between the means of the various fields in each slide In the first method we compared each mean with each of the other means from the same film, adopting the criterion for significance suggested by Price-Jones (1933), namely, that the difference between the two means should be three times the standard error of that difference (calculated as the root of the sum of the squares of the standard error of each mean) The number of pairs of means in the 10 cases investigated varied from 66 (in the case where there were 12 fields) to 10 (in the case where there were 5)

In four cases, in which there were, respectively, 10, 10, 21 and 66 pairs of fields for comparison, there was in no instance any significant difference between any two pairs , of the rest, the number of pairs in which there is and is not a significant difference are as follows —

Number	Total number of pairs	Difference significant	Difference not significant
43	36	9	27
B	21	8	13
29	21	5	16
55	15	6	9
33	21	7	14
44	15	6	9

The other method that we adopted was working out Fisher's ' t ' for each individual field by the formula —

$$ `t` = \frac{d}{\frac{s}{\sqrt{n}}} $$

where ' d ' is the difference between the mean size of the red cells in the field and the mean size of the red cells in the whole film (assuming that the mean of the 500 cells measured is the mean size of the cells of the whole film), s = the standard deviation (the root of the variance divided by $n-1$, not by n) and n the number of cells in the field , that is, $\frac{s}{\sqrt{n}}$ is the standard error

Table IV shows how the fields in each slide are arranged according to the probability of their being fair samples of the cells of the slide in which they occur —

TABLE IV

Cases \ P =	0 5 +	0 5 to 0 4	0 4 to 0 3	0 3 to 0 2	0 2 to 0 1	0 1 to 0 05	0 05 to 0 02	0 02 to 0 01	0 01 or less	Total fields
D 4	6		2	1	1	2				12
35	3			2		1		1		7
32	2		2	1				1		6
42	3			1				1		5
B	4				1	1	1		1	8
43	1	1	1			1	2	1	2	9
29	2			1		1			3	7
33	1	1	1		1	1		1	1	7
44				1		1			5	7
55	2		1						)	6
TOTALS				52				7	15	74

In one case there is no evidence of the operation of any factor other than the error of random sampling, in three cases there is one field in each which falls outside the 20 to 1 point of probability, and in each of the remaining 6 cases one field or more falls outside the 100 to 1 point of probability. Or, if we consider the 74 fields as a whole, in 52 the mean diameter of the cells does not deviate significantly from the mean of all the cells in the film in which they occur, in 22 the mean is significantly different judged on the 20 to 1 level of significance, and in 15 the mean is significantly different judged on the 100 to 1 level of significance.

Thus, whichever way we judge these figures we must conclude that there is some factor, other than the error of random sampling, operating, and that in the majority of these cases the mean diameters of the red cells in fields in different parts of the same blood film are not equal. What this difference is due to we can only guess, possibly it is due to the way the film is made. e g, to the larger cells being drawn to one end of the film, this is a point we have made no attempt to investigate. The observation does however shake one's confidence a little in the method as a means of ascertaining the *exact* size of red cells in anæmic subjects, as conditions that operate at one part of the film may operate over the whole film and even the counting of 500 cells might not give one an accurate idea of the real size of the cells. Further, it shows that the measurement of cells in one field only may lead to very gross errors, for example, in case 'B' the mean of the red cell diameter in one field deviated from that of the whole 500 cells counted by 0 75μ, admittedly, this was an isolated instance and in the whole series the next largest deviation was only 0 55.

Our object in undertaking this analysis was to see if it would be possible to use the Price-Jones's method as a routine examination for patients under special investigation. We have not timed ourselves accurately, but we do not believe that we have ever taken less than 6 hours to complete the drawing, measurement

and tabulation of 500 cells This makes the method entirely unpiactical for regular use, but we felt that it might be possible by counting a smaller number of cells to get a sufficiently approximate degree of accuracy for practical purposes In very few instances (in our seiies) in any field was the standard deviation more than ± 0 9, so that, employing the formula 't' $= \dfrac{d}{\dfrac{s}{\sqrt{n}}}$ and taking 't' as 2, which for numbers

above 30 means that $P = 0\ 05$ (i e , 20 to 1), we find that d, the deviation of the mean of the sample from that of the population, would not be greater than ± 0 254 when 50 cells weie measured, oi ± 0 180 when 100 cells were measured An error of ± 0 254 would not be a serious one in most instances and from a reading with only this error one could certainly see if a blood was either definitely macrocytic or definitely microcytic

However, in view of the fact that the erior of random sampling is not the only error operating, it is appaient that this degree of appioximation cannot be attained by taking the cells from one part of the film only On the other hand, if all the cells in a field are not taken then caie must be exercised about their selection, in view of our obseivation iegarding the diffeicnce between the size of the cells at the centre and at the peripheiy

To summarize, if a reasonable degiee of accuracy is expected from the employment of Price-Jones's method of ascertaining the mean size of the red cells, at least 10 fields should be included and they should be taken at different parts of the film In our series the average number of cells in a field of 7 5 cm radius was less than 100, but we can take this figure as an average foi the sake of discussion If the degree of accuracy required will be achieved by measuring about 100 cells, then a circle with a radius of 2 5 cm must be drawn in the centre of the field the magnification adjusted to a thousand times for the diameter of this circle only (it will not be the same as foi the whole field) and the cells falling within this circle only drawn and measured , if there are 100 cells within a circle of 7 5 cm there will be 11 within an aiea of 2 5, so that ten fields would give over a hundred cells If a greater degree of accuracy is required and 500 cells are to be counted, the circle could be increased to a radius of 5 2 cm and the magnification again adjusted for the diameter of this larger circle

The great value of the Piice-Jones's method of cell measurement is that it gives not only the mean but the dispersion around that mean In certain circumstances this information is required, but for the diagnosis of a case of anæmia it is seldom a matter of great importance and as it absorbs so much time it cannot be adopted as a routine measure in clinical practice we therefore found it necessary to explore other methods of ascertaining the size of red cells

THE PACKED-CELL METHOD OF ESTIMATING CELL VOLUME

When we started our investigation this method had not come into general use It was referred to in most of the American books on laboratory technique, but at that time British workers weie scarcely using it at all We felt that there must be some reason for this hesitation to adopt a method which seemed to be based on common sense, but so fai we have not been able to discover what the objection

to it was   However, during the last two years more and more British workers have adopted this method

One of the first difficulties is the anti-coagulant   Heparin and hirudin are the most satisfactory, but they are expensive and therefore cannot be used for routine work   A convenient anti-coagulant is potassium oxalate   This salt, however, causes a contraction in the red cells and if it is used a ' factor ' must be introduced into the calculations to obtain the true cell volume

The ' normal ' corpuscular volume in Indians

Unfortunately in our earlier investigations which have been reported in this *Journal* (Napier and Das Gupta, 1935) we relied on the halometric readings and did not estimate the cell volume   In view of this fact we thought that we ought to supplement our observation on ' normal ' individuals, reported in the above-mentioned papers, by making observations on the ' normal ' cell volume in Indians The subjects were nearly all doctors, some being members of the staff of the school, others post-graduate students   A few laboratory attendants and menial servants were included   Our original intention had been to include at least 50 persons in this series but circumstances (the departure for Europe of the junior writer) made it necessary to limit the series to 30   We used potassium oxalate as the anti-coagulant, 2 mg per cubic centimetre of blood , the exact method we adopted was as follows —

We prepared a 10 per cent solution of potassium oxalate and of this we placed 0 1 c c into each of a number of 25-c c Erlenmeyer flasks , these we placed for a short time into a hot air sterilizer until the water had evaporated and 10 mg of dry oxalate was left in each tube   These flasks can be kept for almost any length of time , when the estimation is to be made exactly 5 c c of blood is added to the flask which is then placed flat on a table and a circular movement maintained for a few minutes in order to ensure complete mixing   The blood is then transferred rapidly to a graduated tube suitable for placing in a centrifuge   The tubes we used were the graduated tubes supplied with the Hellige normal hæmometer, or smaller tubes which contain 2 c c of blood and are graduated to 0 02 c c —the latter will go into any centrifuge, the former require a deep bucket   The exact amount of blood in the tube must be noted before centrifugalization as a certain amount of evaporation may take place   It is also important to put in a rubber cork to prevent this as far as possible   The tube is placed in a centrifuge doing at least 2,500 revolutions per minute and centrifuged until the column of red cells remains constant , little change will be found to take place after twenty minutes but we usually centrifuged for an hour   The reading is then taken and the cell volume recorded as a percentage of the total blood volume, this is the uncorrected volume and has to be multiplied by a factor

The degree of shrinkage of the red cells on addition of oxalate —There seems to be some difference of opinion between different observers on this important question Osgood (1926) says that with this amount of oxalate (2 mg per c c of blood) the cells shrink 3 5 per cent, whereas Wintrobe (1932) gives the figure as 8 2 per cent

In order to test this point we carried out a number of double estimations with heparin or hirudin and oxalate   It is assumed that neither heparin nor hirudin causes any contraction in the size of the red cells   Using heparin as the control

ın 7 cases we got a mean figure of 6 86 per cent for the shrınkage wıth oxalate, and ın 10 cases where hırudın was used ın the control estımatıons 9 19 per cent If the 17 cases are taken together, 7 94 per cent ıs the shrınkage   Thıs ıs very close to Wıntrobe's figure of 8 2 per cent, and as our numbers were small we have accepted Wıntrobe's figure and have taken 1 09 as the factor by whıch we multıply our readıngs   The figures we obtaıned wıth hırudın, heparın and oxalate are gıven ın tabular form ın Table V —

TABLE V

Serıes	'Heparın'	'Hırudın'	Both
Number ın serıes	7	10	17
Mean hæmoglobın ın grammes	15 730	15 800	15 770
Mean red blood corpuscles ın mıllıons	5 600	5 512	5 552
Cell volume per cent			
Oxalated	46 010	47 260	46 740
Oxalated × 1 09 (1 e , corrected)	50 150	51 510	50 950
Heparın or hırudın	50 050	50 740	50 450
Mean corpuscular volume ın cubıc μ			
Oxalated (corrected)	89 550	93 450	91 770
Heparın or hırudın	89 380	92 050	90 870
Mean corpuscular hæmoglobın ın $\gamma\gamma$	28 090	28 660	28 400
Mean corpuscular hæmoglobın concentratıon (corrected)	31 370	30 670	30 970

Expressıon of result as absolute figures —The calculatıon of the volume ındex presents exactly the same dıfficulty as does that of the colour ındex, only ın the former case the dıfficulty seems rather more pronounced   The normal cell volume ıs varıously reported by Campbell (1922) as 40 per cent, by Gram (1921) as 42 per cent, by Haden (1932) as 45 per cent, by Capps (1903) as 48 per cent and by Larrabee (1911) as 50 per cent, to quote a few wrıters , there ıs also a consıderable varıatıon ın the figures gıven for the normal red cell count   We have obtaıned a hıgher figure than all these, vız , 50 6 per cent, but then our figure for red cells, vız , 5,540,000, ıs also hıgher than the figure usually gıven   The expressıon 'volume ındex' thus becomes meanıngless unless at the tıme the standards of normalıty on whıch the figure ıs based are also gıven   As thıs ıs lıable to lead to confusıon we have decıded to adopt a plan that seems to be gaınıng popularıty and to gıve our results as absolute quantıtıes, the mean cell volume ın cubıc mıcrons, and the mean corpuscular hæmoglobın ın mıcro-mıcro-grammes   A thırd quantıty, *the mean corpuscular hæmoglobın concentratıon, ıs necessarıly a percentage figure*

Dıscussıon on findıngs —Our findıngs ın the 30 cases are analysed ın Table VI There are one or two poınts that seem to call for comment   In the first place, both the hæmoglobın percentage and the red cell count are hıgher ın thıs serıes than ın the prevıous serıes   The dıfference ın the hæmoglobın ın the two serıes ıs definitely 'sıgnıficant'   The method we used was the same ın each case , the populatıons examıned must therefore be dıfferent   Actually, the two samples were drawn from much the same source, but ın the present serıes there were only two menıals, both of whose hæmoglobın percentage happened to be hıgh , ın the other serıes there were 16 menıals ın a total of 50, and theır ınclusıon was an ımportant

factor in lowering the mean hæmoglobin level in the series In the present series the mean red cell count was distinctly higher though the difference is not 'significant' statistically The mean corpuscular hæmoglobin for each individual count was not calculated in the first series, but the mean of the whole series was 27 56 $\gamma\gamma$, in the present series it is 28 53 $\gamma\gamma$ with a standard error of $\pm$ 0 422, it is apparent therefore that the difference between these two means could not be 'significant' unless the standard error of the first series happened to be less than $\pm$ 0 254, a very unlikely contingency (*vide* note by Dr Raja)

TABLE VI

Hæmatological findings in 30 normal Indian males

	Mean	Maximum	Minimum	Standard deviation
Hæmoglobin in grammes per 100 c c of blood	15 700	17 32	13 02	± 0 91
Red cells per c mm in millions	5 533	6 88	4 72	± 0 49
Volume of packed cells percentage of whole blood—oxalate method corrected	50 530	55 60	44 10	± 3 02
Mean corpuscular volume in cubic microns = cu μ	90 490	105 30	72 70	± 7 90
Mean corpuscular hæmoglobin in micro micro grammes = $\gamma\gamma$	28 530	32 60	21 60	± 2 31
Mean corpuscular hæmoglobin concentration in per cent	31 070	34 10	28 60	± 1 20

The second point is that the mean corpuscular volume is rather higher than the figure given as normal by British and American workers, and as a corollary the hæmoglobin concentration is distinctly lower Wintrobe's (1932) figures are —

Mean corpuscular volume   87 cubic μ
Mean corpuscular hæmoglobin   29 577 $\gamma\gamma$
Mean corpuscular hæmoglobin concentration   35 per cent

The possibility that the blood samples were incompletely centrifuged has to be considered However, an efficient electric centrifuge was used and centrifugalization was usually continued for at least an hour from time to time the samples were placed in a larger centrifuge which attains a speed of 10,000 revolutions per minute and no further settlement was ever noticed, so that any error due to the centrifuge factor was reduced to a minimum We have subsequently used the same centrifuge, turning at the same speed, for all our estimations in anæmic patients. The question of the effect of the speed of the centrifuge on the completeness of packing is one that needs further investigation We do not consider that it is quite sufficient to say that centrifuging must be continued until no further settlement occurs

It is interesting to note that although the coefficient of variation of the hæmoglobin estimations and packed-cell volume are 5 80 and 5 98, respectively, the coefficient of variation of the mean corpuscular hæmoglobin concentration is only 3 86, this is apparently a fairly constant factor in health

Cell-volume estimations in 'normal' Assam tea-garden coolies

Similarly, in our normal standards for tea-garden coolies we did not include cell-volume estimations Circumstances did not permit us to repair this deficiency, in an entirely satisfactory manner

We were not ourselves able to undertake another series of controls, the few that we did would be of no value as the estimations were mostly made when we were testing the method in general, and the centrifuge in particular In our investigations in anæmia cases, we used the packed-cell method of estimating the size of the red cells, and many of the patients were followed up for some weeks after their blood counts had returned to figures that were within the normal range for the coolie population, in 34 cases the hæmoglobin was within or above the 'normal' range, that is to say, it was not less than 10 grammes in men or 9 grammes in women in these cases the treatment had been concluded at least one month and in some instances $2\frac{1}{2}$ months when the estimation was made The figures for these 34 cases are given in Table VII —

TABLE VII

Hæmatological findings in 34 tea-garden coolies

		Maximum	Minimum	Mean	Standard deviation	Combined mean and standard deviation
Hæmoglobin in grammes per 100 c c	M	14 85	10 31	12 63	± 1 42	11 96
	F	13 75	9 35	11 30	± 1 19	± 1 45
Red cells in millions per c mm	M	6 43	4 58	5 27	± 0 71	5 10
	F	5 96	3 50	4 93	± 0 61	± 0 64
Cell volume percentage, corrected	M	42 00	32 15	36 63	± 3 46	35 73
	F	49 05	25 07	35 10	± 5 34	± 4 76
Mean corpuscular volume in cubic microns	M	95 08	61 01	71 29	± 7 04	71 88
	F	89 76	55 51	72 30	± 8 80	± 8 13
Mean corpuscular hæmoglobin in micro micro grammes	M	28 14	21 23	23 93	± 2 31	23 59
	F	27 63	18 33	23 35	± 2 40	± 2 38
Mean corpuscular hæmoglobin concentration per cent	M	38 37	27 70	32 50	± 3 10	32 74
	F	38 54	22 79	33 07	± 2 55	± 2 88

In this series the mean cell volume is strikingly low, the mean cell hæmoglobin is also low, but the hæmoglobin concentration is about normal These patients had all received a thorough course of iron which seems to have brought the concentration of hæmoglobin in each cell up to capacity, therefore the low hæmoglobin content is due to some other factor that influences the size of the cells It is obvious that these figures cannot be accepted as 'normal' even for the coolie population, and further investigations will have to be made

Summary and Conclusions

Different methods of measuring red cells are discussed

In the writers' experience, the halometric method is of no practical value The opinion is expressed that the method is responsible for many errors in diagnosis in this country

The Price-Jones's method giving as it does not only the mean size of the red cells but the dispersion around that mean, is a valuable and accurate method, but is extremely laborious The method was used in 10 cases of anæmia and the results were analysed certain fallacies that do not seem to have been discussed by other writers were noted The possibility of reducing the labour involved sufficiently to bring the method within a practical sphere is discussed and a procedure, suggested by the writers' experience, is outlined The method is not however considered to be a practical one for general use, even for a research laboratory

The packed-cell method of estimating cell volume is discussed The method adopted by the writers is described They used potassium oxalate (0 2 per cent) as the routine anti-coagulant, and in a trial in 17 cases, in which hirudin or heparin were used in the controls, they found a shrinkage of 7 94 per cent In view of this finding they have accepted Wintrobe's figure of 8 20 per cent, and have adopted his procedure of multiplying the cell-volume readings by the factor 1 09

The cell volumes, together with the total red cells and the hæmoglobin in grammes per 100 c c, of 30 apparently-healthy city-dwelling male Indians were estimated, and the results, expressed in absolute figures as the mean corpuscular volume, the mean corpuscular hæmoglobin and the mean corpuscular hæmoglobin concentration, are summarized in tabular form The mean and standard deviation for these three quantities was worked out, and it is shown that the mean corpuscular volume is higher than the British and American standards, and that the mean hæmoglobin concentration is lower On the other hand, the cell volume amongst the Assam coolie population is strikingly low, but in this case the subjects were not unselected, being recovered anæmic patients, this point will have to be investigated further

The general conclusion arrived at is that the packed-cell method of estimating cell volume is a simple and accurate one, and absorbs practically none of the worker's time, it supplies nearly[+] all the information regarding the size of the red cells that is required in a routine blood examination The fallacies, other than those connected

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[+] It has been shown that in acholuric jaundice the red cells are thicker than normal and a similar or opposite change may occur in other diseases, to show this, both the mean diameter and the mean corpuscular volume would have to be ascertained

with inaccuracies in the graduation of the tubes, are due to the shrinkage of the cells on addition of the anti-coagulant and to the speed of the centrifuge, the former can be overcome by using a constant quantity of oxalate and multiplying the reading by a factor, and by using the same centrifuge at the same speed for all one's estimations, both in normals and in anaemic patients, errors due to the latter can be reduced to a minimum   The question of the effect of speed of centrifugalization seems to require further investigation

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[*Note*—As neither writer claims to be an authority on statistical methods they handed their paper to Dr K C K E Raja, Officiating Professor of Statistics at the All India Institute of Hygiene and Public Health, Calcutta, for his opinion his report is given below
Both the sentences that Dr Raja criticizes have now been modified to meet his criticism —L E N]

(*Note by Dr K C K L Raja*)

1   On page 982, it is stated that the image of the red blood cell at the periphery of the field is $\frac{\sqrt{25 + (7\,5)}}{25}$ or 1 044 times the size of the image of a cell at the centre   I have consulted Prof P C Mahalanobis, Professor of Physics at the Presidency College, and am informed that the image at the periphery will be larger than at the centre but, in the case of a complex system of lenses as in a microscope, the relationship in size between the two images cannot be expressed so simply as you have done   The makers will probably be able to provide the necessary information for calculating the enlargement in size   Perhaps, for your purpose, it is enough to indicate that the sizes will not be equal and leave out the estimation of the enlargement at the periphery

2   On page 988 it is stated that 'the mean corpuscular haemoglobin for each individual was not calculated in the first series, but the mean of the whole series was 27 56 $\gamma\gamma$, in the present series it is 28 53 $\gamma\gamma$ with a standard

error of $\pm$ 0 422 , it is apparent therefore that the difference between these two means cannot be "significant"'

Evidently, the standard error of $\pm$ 0 422 relates to the mean of the second series and not to the difference of the means of the two series   The statement that this difference, namely 0 97, is not significant can be true only if the standard error of the mean of the first series does not fall below $\pm$ 0 2538   This is on the assumption that the same samples in the two series are of fairly large size and taking twice the standard error as approximately representing the 5 per cent level of significance

Except for the two remarks made above I consider that the conclusions arrived at have been based on a satisfactory statistical treatment of the data

<div align="right">

K C K E RAJA

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THE USE OF TAPIOCA IN IMMUNIZATION WITH SNAKE VENOMS

BY

CAPTAIN S M K MALLICK M B , M R C S , M R C P D P H , I M S

Inquiry under the Indian Research Fund Association

(From the Central Research Institute)

THE immunization of horses for the production of antivenene has been carried out at this Institute for over thirty years by the use of solutions of the dried venoms of the Indian cobra and the daboia, unaltered by any method of detoxication or by the addition of any substance employed on account of its (non-specific) adjuvant action

The ordinary course of immunization employed for many years consisted in the injection of progressive doses of the two venoms at 10 days' interval commencing with a dose of 0 5 mg of each , typical examples of the dosages employed would be as follows —

> 0 5 mg , 1 mg , 2 mg , 4 mg , 8 mg , 16 mg , 16 mg , 25 mg , 40 mg , 55 mg , 70 mg , 85 mg and 100 mg of each venom

1,500 units antitetanic serum was usually added to each dose after a level of 10 mg was reached and with high dosage antivenene was also given Such a series of injections was usually found necessary to obtain the minimal titre required, viz , 1 c c of the unconcentrated serum to neutralize 0 5 mg of cobra venom and 1 0 mg of daboia venom

The Table shows examples of the dosage and the period of immunization required to obtain the minimal titre by that method in 12 of the horses used for serum production It will be seen from the Table that in the majority of cases it was necessary to employ doses as high as 100 mg of each venom in order to reach titre, and the total dosage required varied from about 300 mg to 400 mg

The period of immunization to reach titre was usually 15 to 17 weeks In the case of certain horses a longer period of immunization was necessary and a higher total dosage was required Horse No 12 shown in the Table took nearly a year to bring up to minimal titre and received doses up to 250 mg and a total of 2,744 mg of each venom

Methods for the improvement of immunization of the horses have been under study and the use of tapioca and of an adsorbent suspension of aluminium hydroxide has been tried both in the practical production of antivenene from horses, and in the experimental study of their comparative value on a small scale in goats Ramon (1931) has shown that the addition of tapioca to tetanus toxoid has been of great

value in the production of antitetanic serum and he attributes this to the production of local inflammatory œdema with exudation which delays absorption and prevents the rapid elimination of the antigen   The use of tapioca was tested in the course of immunization of two fresh horses in the first instance   A sterile suspension of tapioca in the proportion of 6 per cent was added to the immunizing dose of venom and a reduced dosage employed

With this addition the weekly dosage of the two venoms employed was of the following order —

0 5 mg , 1 mg , 2 mg , 3 mg , 4 mg , 5 mg , 6 mg , 8 mg , 10 mg , 15 mg , 20 mg , 25 mg and 30 mg

With this dosage one horse which had not previously been immunized reached full titre in 13 weeks with a maximum dose of 25 mg and a total of 99 mg of each venom, while the other reached titre in 14 weeks with a maximum of 30 mg and a total of 129 mg of each venom   Although the total period of immunization was not greatly reduced the quantity of venom required was reduced to one-third

In the older method of immunization large abscesses were frequently experienced with the high dosage used, but with the smaller doses and the addition of tapioca such abscesses were much less frequent although there was a considerable degree of local swelling   The horses kept better condition which was no doubt a factor in the effective production of antitoxin   The results of the use of tapioca in immunization of fresh horses have led to its employment in the maintenance of the titre of older horses some of which have been under immunization for 5 or 6 years   In the case of these horses it has been found that the dose necessary to maintain titre has been gradually reduced   For example, in the case of one horse it had been found necessary to raise the dose of each venom to 150 mg , after resting it, to reach titre again while when tapioca was added it could be maintained at titre with a dose of 40 mg to 50 mg   In addition, it has been found that horses maintain titre for longer periods than formerly when injections are stopped and comparatively small doses will keep titre at a suitable level

There has never been any difficulty in obtaining high anti-daboia venom titre and horses frequently showed titres up to 5 times that of the minimal standard laid down for this venom   In the case of anti-cobra titre, difficulty has been found in getting a high figure with the older methods of immunization, but with the use of tapioca much better results are now being obtained   The serum of one horse, for example, which had not previously passed the minimal standard of 1 c c neutralizing 0 5 mg gave a titre of 1 c c neutralizing 1 2 mg when the immunization was continued with addition of tapioca

A trial of the immunizing value of the venoms with and without the addition of tapioca or aluminium hydroxide suspension was carried out in a series of six goats of approximately equal weight, the goats employed being immunized as follows —

(i)	Goat No	1	Cobra venom only
(ii)	,,	2	Cobra venom and tapioca
(iii)	,,	3	Cobra venom and aluminium hydroxide
(iv)	,,	4	Daboia venom only
(v)	,,	5	Daboia venom and tapioca
(vi)	,,	6	Daboia venom and aluminium hydroxide

The dosage of venom in each case was a weekly injection of the following progressive quantities —

0 5 mg , 1 mg , 2 5 mg , 5 mg , 7 5 mg , 10 mg , 12 5 mg , 15 mg and 17 5 mg

The results obtained in the animals differed considerably in regard to reaction to the cobra venom

Goat No 1 receiving this venom without any addition died after the fourth day and a second goat used in replacement lost weight and died after 11 injections without reaching titre

Goat No 2 receiving cobra venom and tapioca reached titre after 11th dose (22 5 mg cobra venom) and was in good condition

Goat No 3 receiving cobra venom and Al(OH)$_3$ died after 5th injection This one was replaced and did not reach titre by the 12th injection and died of other causes

In the case of the three goats immunized with daboia venom all reached equal titre on the same day after nine injections   The goat receiving tapioca along with the venom showed a lesser degree of local reaction than those to which this addition had not been made   The use of tapioca is thus shown to have a marked advantage in the course of immunization with cobra venom, animals standing immunization much better than without this addition and coming up to titre rapidly   The use of tapioca has marked advantages during immunization with daboia venom

TABLE

Showing dosage and the period of immunization to obtain minimum titre in horses

Horse number	Period of immunization	TOTAL DOSAGE		MAXIMUM DOSE	
		Cobra venom	Daboia venom	Cobra venom	Daboia venom
1	15 weeks	406 5	406 5	100	100
2	15 „	406 5	406 5	100	100
3	20	363 5	363 5	95	95
4	12	235 5	235 5	80	80
5	20 „	363 5	363 5	95	95
6	13	336 5	336 5	100	100
7	17 „	335 5	335 5	140	140
8	17 „	310 5	310 5	90	90
9	17 „	310 5	310 5	90	90
10	21 „	715 5	715 5	145	145
11	22 „	526 5	526 5	80	80
12	50 „	2,744	2,744	250	250

SUMMARY

1   The addition of a sterile suspension of tapioca and of aluminium hydroxide to the venom solutions employed in immunizing animals for the production of antivenene was tested

2   When tapioca suspensions were added it was found possible to bring horses to standard titre against both *cobra* and *daboia* venoms with greatly reduced dosage of the venoms, the quantities employed being one-third of that found necessary without this addition

3   Comparative trials in a series of goats showed that the addition of tapioca was of special value in immunization with cobra venom and that this addition was more satisfactory than the use of aluminium hydroxide

The extensive suppurative reactions with abscess formation, which occurred when tapioca was not employed and with the larger doses formerly used to reach titre, were not observed when tapioca was added or were of much lesser degree

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STUDIES ON INDIAN SNAKE VENOMS

Part I.

DABOIA VENOM ITS CHEMICAL COMPOSITION, PROTEIN FRACTIONS AND THEIR PHYSIOLOGICAL ACTION

BY

S N GANGULY, PH D (Lond),

AND

M T MALKANA, M Sc

Inquiry under the Indian Research Fund Association

(From the Central Research Institute, Kasauli)

CHEMICAL ANALYSIS OF THE DABOIA VENOM

A PRELIMINARY investigation into the nature of the chemical elements present in the daboia venom led to the identification of the following   C, H, N, S and O , with traces of inorganic substances and colouring matters

The complete absence of phosphorus, as indicated by Neumann's test (using casein as a control), and its further confirmation by the standard method of combustion with metallic sodium, proved the non-existence in the venom of substances such as lecithin, kephalin and nucleoproteins

Since a solution of the venom gave all the characteristic tests for proteins it was further examined with a view to identify the various proteins present   Using the standard methods only globulins, albumins and substances of the nature of secondary proteoses were found to be present   Absence of any protein in the supernatant fluid left after full saturation with ammonium sulphate showed the non-existence of peptones

The total nitrogen content of the dried venom, as estimated by a modified micro-Kjeldahl method was found in successive tests to be 15 7 per cent, 15 6 per cent and 15 7 per cent Out of this total, nitrogen from the precipitate obtained by treating a venom solution (1 in 100) with an equal volume of 50 per cent trichloracetic acid was found in successive tests to be 15 5 per cent showing thereby that the dried venom contains 96 8 per cent (N × 6 25) of proteins Non-protein nitrogen, however, as determined from the supernatant fluid left after precipitating the venom solution with trichloracetic acid was found to be as low as 0 18 per cent (i e , within the limits of experimental error)

A small fraction of the venom, probably of the nature of lipoids, could be isolated by extraction with ether in a Soxhlet's extraction apparatus Its quantity was estimated to be 2 8 per cent of the dried venom , but as on testing its toxicity it was found to be physiologically inert, no further attempt was made to determine in more detail the chemical nature and identity of this ether-soluble substance

DETERMINATION OF THE PROTEIN FRACTIONS OF THE VENOM

(1) The proteins of the venom were fractionated by the method described below —

*Estimation of globulin —*The venom solution (10 c c of 1 in 100) was saturated with sodium chloride and left in the refrigerator overnight The following morning the precipitate obtained was separated from the supernatant fluid by centrifuging and washing several times with a saturated solution of brine The nitrogen content of the precipitate representing the globulin fraction of the venom was estimated It was found on successive tests that the globulin fraction contained an average of 3 7359 per cent of nitrogen in relation to the dried venom This represents 23 35 per cent of globulin (N × 6 25)

*Estimation of albumin and proteoses —*The venom solution (10 c c of 1 in 100) was saturated with sodium chloride and left in the refrigerator overnight The following morning about 5 c c to 6 c c of the clear supernatant fluid was pipetted off carefully An aliquot part of this globulin-free solution (containing albumin and proteoses) was estimated for its nitrogen content while another part was heated to boiling point for two minutes to effect a complete coagulation of the albumin, leaving the proteoses behind in solution The nitrogen contents of the precipitate and the filtrate were separately estimated The results showed that the albumin and proteose respectively formed 22 12 per cent and 50 52 per cent of the venom

The estimation of nitrogen of each fraction was carried out by a modified micro-Kjeldahl method using a mixture of concentrated sulphuric acid, potassium sulphate and a trace of copper sulphate as the digestion mixture An appliance was devised to produce a constant flow of ammonia-free air through the mixture during the process of driving off the liberated ammonia from the digest for estimation by absorption in 0 01 N sulphuric acid, and subsequent titration by 0 01 N caustic soda of the amount of acid consumed by the liberated ammonia.

The composition of the venom was thus found to be —

Protein (total)	96 80	per cent
Globulin	23 35	,,
Albumin	22 12	,,
Albumin + proteoses	72 74	,,
Proteoses	50 52	,,
Lipoids	2 80	,,

LOCATION OF THE ACTIVE PRINCIPLES IN THE VENOM FRACTIONS

The tests of the physiological action of the fractions of the venom were carried out in regard to —

(a) *Lethal action in pigeons as determined by the effect of intravenous injection* — This is a routine test in use at the Institute and the average sample of the dried daboia venom in use has an m l d of 0 01 mg for a pigeon of 300 grammes weight The dose of the venom fractions used is expressed as the equivalent of original dried venom   This test is taken in indicating the presence of neurotoxin

(b) *Coagulant action* —The method of test employed was that used by Taylor and Mallick (1935)

(c) *Presence of hæmorrhagin* —The test used was that of Pratt-Johnson (1934) by intradermal injection in white rabbits, the *hæmorrhagin* dose of dried daboia venom being 0 1 mg

(1) *Neurotoxin*

The proteins of the venom after precipitation by saturation with ammonium sulphate or acetone were found to contain the full toxicity of the venom as judged by the immediate lethal effect in pigeons, the m l d of protein obtained in this way being the same as the equivalent amount of dried venom

The globulin fraction separated by full saturation with NaCl or 50 per cent saturation with ammonium sulphate was found to be inert, pigeons surviving a dose up to the equivalent of 20 times the m l d

The next step was therefore to ascertain whether the toxicity could be ascribed to the albumin or the proteoses or to both   Since on boiling the venom solution is completely detoxicated it was found necessary to develop a suitable technique for the separation of the proteoses and the albumin in order to test the physiological action of these fractions separately   Various methods such as (i) treatment of the dried venom with 45 per cent alcohol, as suggested by Kellaway (1929) as a possible method for fractionation of death-adder venom and (ii) precipitation with hydro-ferrocyanic acid, etc , were found unsatisfactory owing to the detoxication of the venom as well as to the denaturing of the proteins produced by these reagents

After a number of trials the method which was found satisfactory was exposure of a venom solution (previously freed from globulin by full saturation with sodium chloride) to a temperature of 65°C for six minutes (Acton and Knowles, 1921)   The albumin fraction was almost completely coagulated leaving the proteoses in solution The m l d of this protein fraction calculated in relation to the proportion of original

dried venom which it represented showed that this contained the whole toxicity of the venom   This finding shows that the albumin fraction is not the toxic one

TABLE I

Showing neurotoxic action of the protein fraction of daboia venom

Fractions	Mean percentage by weight in relation to the dried venom	Intravenous dose in mg (equivalent of dried venom) injected into pigeons	Result	REMARKS
1 Total protein	96 80	0 01	Died	Full toxicity
2 Globulin	23 35	0 02 0 05 0 1, 0 2	Survived	Non toxic
3 Albumin	22 12			Denatured on heating
4 Albumin and proteoses	72 74	0 01	Died	
5 Proteoses after boiling No 4	50 52			Detoxicated on heating
6 Fraction of globulin free venom solution not coagulable at 65°C in six minutes (proteoses)	56 59	0 01	Died	Full toxicity
7 Residue after ether extraction		0 01		, ,,
8 Ether soluble lipoids	2 80	0 05, 0 1	Survived	Non toxic
9 Globulin free fraction coagulable at 65°C (albumin)		0 02, 0 05, 0 1		

(2) *Hæmorrhagin*

Intradermal injections of a series of doses of solutions of the fractions prepared as already described were given to white rabbits on the skin of which the *hæmorrhagin* reaction could easily be seen

The following are the results of a series of tests —

		Result
Daboia venom (control)	0 1 mg	+ +
Globulin	0 1 mg , 0 2 mg	— —
Albumin + proteoses	0 1 mg	+ +
Proteoses . , , ,	0 1 mg	+

The proteoses fraction is shown to contain the *hæmorrhagin* of the venom   The more intense reaction in the case of the combined albumin and proteoses fractions is due to the fact that the *hæmorrhagin* is to a certain extent heat-labile and in the process of coagulation of the albumin by heating at 65°C   for six minutes the *hæmorrhagin* content of the proteoses is somewhat reduced

(3) *Coagulant action*

Coagulation tests were carried out on sheep's blood which had an average coagulation period at a temperature of 23°C of 15 minutes, by the technique used Solutions of the venom fractions were used equivalent to 1 in 10,000 of dried venom, 0 1 c c being added to 0 9 c c of freshly drawn sheep's blood, the final dilution being equivalent to 1 in 100,000

The results of the tests are shown in Table II —

TABLE II

Showing coagulant action of the protein fraction of daboia venom

Fractions		Coagulation time	
		Minutes	Seconds
1	Total protein	1	33
2	Globulin	13	25
3	Fraction of globulin free venom solution not coagulable at 65°C in six minutes (proteoses)	1	25
4	Residue after ether extraction	1	35
5	Ether soluble lipoids	9	10
6	Globulin free fraction coagulable at 65°C (albumin)	14	40

The proteoses are thus shown to also be the coagulant fraction of the venom

ATTEMPTS ON THE SEPARATION OF THE ACTIVE PRINCIPLES OF DABOIA VENOM

It has been previously shown that the *hæmorrhagin* fraction can be destroyed in the venom, leaving the coagulative and neurotoxic factors, by heating the venom

solution at a temperature of 65°C for over six minutes The separation of the coagulative and neurotoxic fractions by several methods was attempted

Adsorption methods—For this work the adsorbents tried were   (1) kaolin, (2) keiselguhr, (3) barium sulphate, (4) Fuller's earth, (5) charcoal (norit), (6) Willstratter's aluminium hydroxide C and (7) barium carbonate   Barium carbonate was used in view of the claim of Holden (1933) to have removed the coagulative factor from the venom of certain Australian snakes with this adsorbent

The method employed was as follows   0 2 gramme of each adsorbent was intimately mixed with 10 c c of dabora venom (of dilution 1 in 1,000) and kept in a refrigerator overnight   Subsequently the supernatant fluid was separated by centrifuging and tested for its coagulative and neurotoxic action   The adsorption experiments were all done at pH 7 2

The results are shown in Table III —

TABLE III

Showing effect of adsorbents on coagulative and neurotoxic action

Adsorbent used	Coagulation time		Amount equivalent in mg of the dried venom injected into pigeons	Result
	Minutes	Seconds		
(1) Kaolin	2	15	0 02	Pigeons died in 2 to 3 minutes
(2) Keiselguhr	2	10	0 02	
(3) BaSO₄	2	10	0 02	
(4) Charcoal	16	35	0 02, 0 05, 0 1	Survived
(5) Fuller's earth	2	7	0 02	Pigeons died in 2 to 3 minutes
(6) BaCO₃	11	10	0 02, 0 05, 0 1	Survived
(7) Dabora venom control	1	15	0 02	Pigeons died in 2 to 3 minutes
(8) Saline control	18	45		
(9) Aluminium hydroxide	10	23	0 5	Survived

Only barium carbonate, charcoal and aluminium hydroxide were found to be effective adsorbents and an adjustment of reaction at ranges between pH 3 and pH 10 was not found to effect adsorption

In the case of barium carbonate further work was done on the elution of the venom after adsorption, the details of the process being as follows —

Daboia venom 10 c c (of dilution 1 in 100) was added to freshly prepared $BaCO_3$ made by passing a stream of CO_2 through a saturated solution of $Ba(OH)_2$ (1 7133 g = 1×10^{-5} g molecules of $BaCO_3$ per c c) at a temperature below 10°C till the solution was acid to litmus   The total solution was made to a volume of 100 c c by adding distilled water (making up the venom dilution of 1 in 1,000) The mixture was thoroughly shaken and then left in the refrigerator overnight The clear supernatant fluid was separated from the $BaCO_3$ by centrifuging and kept for testing separately for the neurotoxin as well as the coagulant in the usual way

The solid $BaCO_3$ residue, as separated from the supernatant fluid by centrifuging, was washed once thoroughly with water and subsequently separated by centrifuging   To the washed solid was added 100 c c of a mixture of KH_2PO_4 buffer (pH 6 9) and 25 per cent glycerine   After being kept in contact for 2 to 3 hours the solid $BaCO_3$ was separated by centrifuging and the clear elution was tested as usual

The results are shown in Table IV —

TABLE IV

Showing results of adsorption of venom solution with $BaCO_3$ and its elution from the adsorbents with phosphate

Solution tested	Coagulation time		Amount equivalent in mg of the dried venom injected into pigeons	Result
	Minutes	Seconds		
Supernatant fluid left after adsorption with $BaCO_3$	21	10	0 02, 0 05, 0 1	Pigeon survived
Elution from $BaCO_3$	1	58	0 01, 0 02, 0 025	1st and 2nd survived 3rd one died]
Daboia venom control	1	35	0 01	Died
Saline control	23	10		

A corresponding adsorption with aluminium hydroxide prepared according to Willstratter's method (Willstratter and Kraut, 1923) was carried out   Ten c c of this $Al(OH)_3$ suspension was mixed thoroughly for 3 to 4 minutes with 100 c c of venom solution (1 in 1,000)   The supernatant fluid was separated by centrifuging and the solid residue treated with a mixture of Na_2HPO_4 and glycerine solution (100 c c) for elution   The results are shown in Table V   With both these methods

effective adsorption and elution was obtained but no separation of the active principles

TABLE V

Showing results of adsorption of venom solution with Al(OH)₃ and its elution from the adsorbate with phosphate

Substance used	Coagulation time		Amount equivalent in mg of the dried venom injected into pigeons	Result
	Minutes	Seconds		
Supernatant fluid left after adsorption with Al(OH)₃	10	23	0 05, 0 1, 0 5	Survived
Eluted	2	0	0 02 0 025	Survived (Died in 2 to 3 minutes)
Saline control	11	0		
Daboia venom control	1	45	0 01	Died

Fractional precipitation with ammonium sulphate —The separation of the active fractions of the venom was also attempted by means of saturation of venom solution with varying percentages of ammonium sulphate

Twenty c c of a 1 per cent solution of venom was mixed with 20 c c of saturated ammonium sulphate solution and left in the refrigerator overnight   The white precipitate that settled down was separated by centrifuging, washed twice or thrice with 50 per cent saturated ammonium sulphate solution and then dialysed for four or five days in running water till it was free from the salt   The dialysed product was dissolved in saline and made up to ten times the original volume   The globulin fraction thus separated was tested for coagulant and neurotoxic action   The supernatant fluid in successive samples was separately saturated in different ranges, 60 per cent, 70 per cent, 80 per cent, 90 per cent and 100 per cent with further addition of saturated ammonium sulphate calculated by the formula (Wadsworth, 1927)

$X = \frac{cv}{100-c}$ where c represents the percentage of saturation required, X, the total volume of saturated ammonium sulphate to be added to v c c of the original venom solution (1 in 100)   Full saturation with ammonium sulphate was done by adding solid $(NH_4)_2SO_4$ The precipitates and supernatant fluids in each fraction were dialysed as before for 4 or 5 days till they showed complete absence of ammonium sulphate   A sample of the untreated venom was similarly dialysed for 4 or 5 days to serve as a control

The coagulative and neurotoxic action of the precipitates and the supernatant fluids between the different range of saturation was tested   Partial precipitation of these fractions occurred at from 60 to 70 per cent of saturation and full precipitation at from 80 to 100 per cent

The neurotoxic and coagulative principles are not separated by this means (*see* Table VI)

TABLE VI

Showing coagulative and neurotoxic action of dabora venom fractions after precipitation with different concentrates of ammonium sulphate

Percentage of satu ration with (NH₄)₂SO₄	COAGULATION TIME						M l d for pigeons (with the precipi- tates)
	PRECIPITATES		SUPERNATANTS		CONTROLS		
	Minutes	Seconds	Minutes	Seconds	Minutes	Seconds	
Untreated control					1	10	Equivalent to 0 1 mg of the original venom did not kill the pigeons
30 per cent	*Nil*	*Nil*	0	55			
40 „	9	28	1	19			
50 „	8	40	1	48			
60 „	3	15	3	18			0 02 mg , pigeons died
70 „	2	4					
80 „	1	35					0 015 mg , pigeons died
90 „	1	33					
100 „	1	33					
Saline control					12	0	
Dabora venom control					1	33	

Precipitation with acetone —Acetone precipitation was also tried for separation of the active fractions   Ten c c of dabora venom (1 in 100) was mixed with 20 c c of acetone , the precipitate obtained was separated by centrifuging and subsequently added to 20 c c of distilled water   The precipitate was separated by centrifuging and the clear supernatant fluid was again precipitated and washed with acetone   All the protein fractions were thus precipitated by means of acetone and the globulin was separated from the albumin and proteoses by taking advantage of the fact that the globulin is insoluble in distilled water, whereas the other two are perfectly soluble   The globulin fraction thus separated was dried and weighed (0 0219 g , 0 0238 g) and dissolved in 200 c c of saline   The solution of the albumin and proteoses in water was also made up to the same volume by addition of water Thus the globulin was found to be present to the extent of 21 9 per cent to 23 8 per

cent of the dried venom   Results of the tests of the fractions are shown in Table
VII —

<div align="center">TABLE VII</div>

*Showing coagulation and neurotoxic action of daboia venom fractions
obtained by precipitation with acetone*

Substance used	COAGULATION TIME		Amount equivalent in mg of the dried venom injected into pigeons	RESULT
	Minutes	Seconds		
Globulin fraction	21	0	0 02, 0 05, 0 1	Survived
Albumin and proteoses fraction	2	40	0 01	,,
			0 015	Died
Daboia venom control	2	10	0 01	,,
Saline control	21	15		

The results again indicate the presence of both coagulative and neurotoxic
fractions in the proteoses   It is noticed, however, that after several contacts with
acetone the venom loses at least a part of its toxic principles

It has not been possible by any of the methods employed to separate fractions
showing different physiological actions

<div align="center">SUMMARY</div>

1   The chemical analysis of daboia venom shows that it contains the elements
C, H, N, S and O   No phosphorus is present and accordingly no such substances
as lecithin, kephalin and nucleoproteins

2   The dried venom contains 15 5 per cent protein nitrogen, indicating 96 8
per cent of protein, and ether-soluble lipoids are present to the extent of 2 8 per
cent

3   The protein fractions are   globulin 23 35 per cent, albumin 22 12 per cent
and proteoses 50 52 per cent

4   The neurotoxic the coagulant and the *haemorrhagic* actions of daboia
venom are attributed to the secondary proteoses

5   It has not been possible to separate from one another fractions responsible
for these actions by means of adsorption methods or by precipitation with ammo-
nium sulphate or acetone

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CIRRHOSIS OF THE LIVER FOLLOWING CHRONIC INTOXICATION WITH CARBON TETRACHLORIDE: AN EXPERIMENTAL STUDY [*]

BY

M V RADHAKRISHNA RAO, M B, B S PH D

(*From the Department of Pathology, Medical College, Vizagapatam*)

THIS experimental work was undertaken mainly to investigate the evolution and nature of cirrhosis of the liver induced by chronic intoxication with carbon tetrachloride, as it was hoped that such a study would contribute to an understanding of the genesis of fibrosis in human cirrhosis

EXPERIMENTAL METHODS

Depending on the reaction of the experimental animal, subcutaneous injections of 0 1 c c to 0 2 c c of pure carbon tetrachloride were given once or twice a week for varying periods (*vide* Table) In this experiment, which lasted for nearly 15 months, 21 albino rats, 6 rabbits and 17 guinea-pigs were used In addition to the subcutaneous injections, 3 albino rats were given daily 10 minims of carbon tetrachloride suspended in milk The drug was administered only by the oral route in all the six rabbits and in 2 albino rats

The animals were kept on a normal laboratory diet (milk, green fodder, germinating giam, etc)

As the object of the experiment was to induce cirrhosis by repeated, sub-lethal injections, the animals were not killed during the early part of the investigation and the observations on the early changes in the liver were mainly based on a study of the animals which died during this period The administration of the drug was suspended in some animals which received a number of injections, in order to study the regenerative changes The liver in all the animals was carefully investigated by the different staining methods mentioned below --

(1) Ehrlich's acid hæmatoxylin and eosin—for routine staining

(2) Weigert's iron hæmatoxylin and van Gieson's stain—for staining connective tissue

_____

(3) Weigert's elastic stain—for staining elastic tissue—counterstained with alum carmine

(4) Prussian blue reaction (sections were treated with potassium ferrocyanide and hydrochloric acid solutions and counterstained with eosin)—for the presence of iron (to detect the presence of hæmosiderin)

(5) Scharlach R—for staining fat—counterstained with Delafield's hæmatoxylin (frozen sections were used)

(6) Foot and Menard's (1927) silver carbonate impregnation method—for staining the reticulum (frozen sections were used)

Observations

General

Guinea-pigs were found to be very susceptible to the drug, as they generally died after the first or the first few injections, and no animal in this series survived to receive more than 10 injections   The albino rats which received repeated injections gradually became wasted lost their hair and died   There was no appreciable change in the general condition of the rabbits which received the drug only by mouth

Sloughing and ulceration at the site of the injection were occasionally observed

Autopsy findings

I   The liver —(*a*) In one instance in which the animal (No 12) died *immediately after* the injection, the liver showed marked congestion of the hepatic and portal venous trees, but the parenchyma showed no noticeable changes

(*b*) In animals which died 12 to 72 hours *after one injection* (8 in this series) the liver was markedly congested and the surface showed a slightly trabeculated appearance (Gardner *et al* , 1925) due to alternating red and slightly yellowish areas

Microscopically, there was marked engorgement of the hepatic and portal venous trunks

In one animal (No 6) which died 12 hours after the injection, the hepatic cords around the central veins were swollen and contained small or big fat vacuoles , their nuclei were pyknotic   The hepatic cords around the portal spaces were normal but those in the middle zone showed slight fatty degeneration   In preparations especially stained by the Foot and Menard's silver impregnation technique, the reticulum of the liver (in this case) was found to be intact

The liver showed marked necrosis of the parenchyma around the hepatic terminals (involving half to two-thirds of the so-called lobules) in both the instances in which the animal survived 24 hours after the injection (Plate XLI, fig 3)   The central veins were generally destroyed and if evident, showed a desquamation or a rupture of their endothelial lining   The sinusoids were congested and the necrotic central zone was invaded by Kupffer cells, polymorphonuclear leucocytes and mononuclear phagocytes   The middle and occasionally the peri-portal zones showed fatty degeneration of the hepatic cords   The liver showed more or less similar changes in the animals which survived two (2 animals) or three days (3 animals)

PLATE XLI.

FIG. 2

Figs 1 and 2 — White rat No 11 $\frac{\text{18 injections and 10 m orally for 168 days}}{\text{277 days}}$

Photograph of the liver showing hobnail roughness of the surface
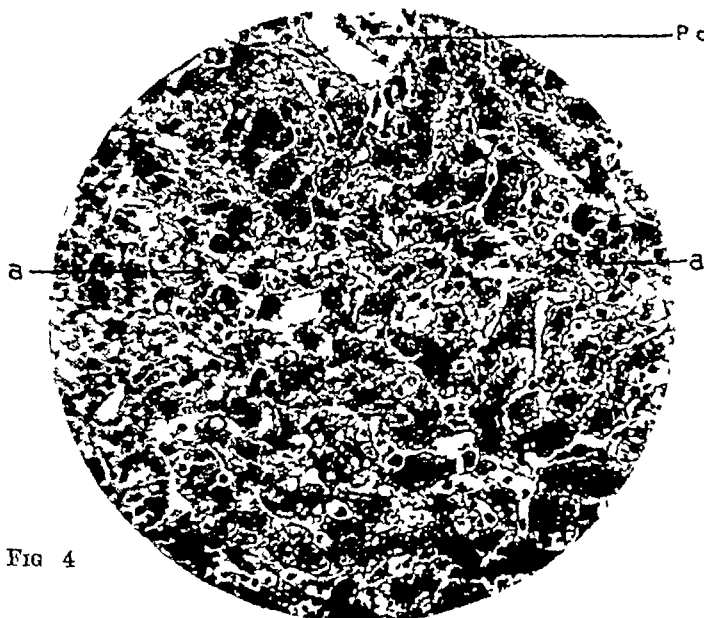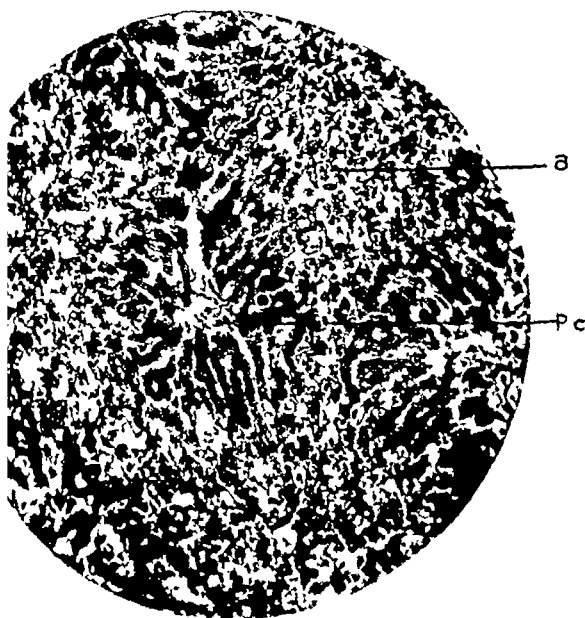
FIG 4

Fig 3 — Guinea pig No 21 $\frac{\text{1 injection}}{\text{1 day}}$

Photomicrograph of a section of the liver showing the necrosis of the parenchyma (a) around the hepatic terminals p c — portal space

(Ehrlich's acid hematoxylin and eosin, apochromat objective—8 mm, periplan o k eye piece—4 mm —Leitz)

Fig 4 — Guinea pig No 20 $\frac{\text{1 injection}}{\text{3 days}}$

Photomicrograph of a section of the liver showing the invasion of the necrotic zone (a) by mononuclear phagocytes and Kupffer cells p c — portal space

(Ehrlich's acid hæmotoxylin and eosin, apochromat objective—4 mm, periplan o k eye piece—4 mm —Leitz)

PLATE XLII
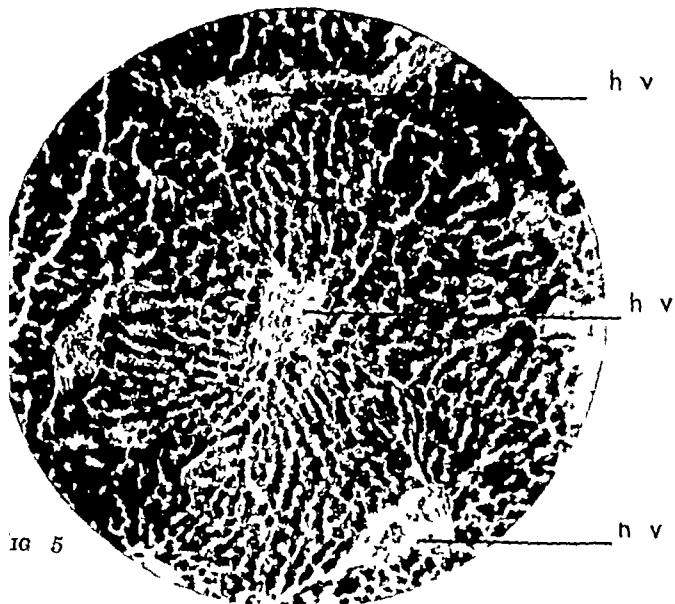
Fig 5 —White rat No 26   7 injections / 17 days

Photomicrograph of a section of the liver showing scarring around the central veins (h v)

(Ehrlich's acid hæmatoxylin and eosin, apochromat objective—8 mm , periplan o k eye piece—4 mm —Leitz)

Fig 6 —White rat No 5   33 injections / 166 days

Photomicrograph of a section of the liver showing irregular, loose stellate areas of fibrosis containing pigmented mononuclear phagocytes around the central veins, and a tendency to pseudo lobulation

(Ehrlich's acid hæmatoxylin and eosin, apochromat objective—8 mm , periplan o k eye piece—4 mm —Leitz)

Fig 7 —White rat No 11   48 injections and 10 m orally for 168 days / 277 days

Photomicrograph of a section of the liver in Fig 1 showing the bands of loose fibrous tissue dividing parenchymal mantle into numerous, rounded islands of varying sizes

(Ehrlich s acid hæmatoxylin and eosin, objective—22 mm , Beck periplan o k eye piece—4 mm —Leitz)

Fig 8 —White rat No 11

Low power photomicrograph of the reticulum of a section of the liver in Fig 1 showing the well marked pseudo lobulation and a deficient reticulum in the pseudo lobules The parenchyma in this section is stained oxalic acid

(Foot and Menard's silver carbonate impregnation technique, objective 22 mm , Beck, periplan o k eye piece 4 mm —Leitz)

but the phagocytosis was more marked (Plate XLI, fig 4) In all these instances the peri-sinusoidal reticular frame work around the hepatic terminals was still intact

(c) Animals which received 2 *to* 6 *injections* (10 animals) showed changes in the liver similar to those described above The granular débris of the coagulated protoplasm around the central veins, which were faintly staining, was invaded by large number of Kupffer cells and mononuclear phagocytes The hepatic cords in the middle zone showed vacuoles of fat in their protoplasm, while those around the portal spaces showed granular protoplasm Evidences of regeneration were not noticed In some instances, the granular débris was cleared off by the phagocytes and the necrosis was limited to a small area around the central vein Occasionally the necrosis was more marked , with the exception of small islands of hepatic cords which showed fine vacuolation with fat, around the portal spaces, the entire parenchymal mantle showed necrotic changes

The central veins were usually not discernible, but, in one instance, the endothelium of the central veins showed desquamation into the lumen

The peri-sinusoidal capillary reticulum around the central veins showed disorganization

(d) The appearances of the liver in the albino rats and guinea-pigs which received 6 *to* 10 *injections* (3 animals) varied considerably In the latter, the appearances of the liver were similar to those described above but, in the former, the liver was atrophic, the surface was slightly irregular and on microscopic examination showed scarring around the central veins (Plate XLII, fig 5), which were dilated and congested The scars were not compact and dense , often they contained large pigmented mononuclear cells The sinusoids around the scarred areas were congested , the hepatic cords around the portal vein were hypertrophic and showed evidences of regeneration

(e) Though the connective tissue was not proportional to the number of injections, animals which received 10 *to* 30 *injections* (3 animals) showed well-marked fibrosis around the central veins The hepatic parenchyma showed no loss of pattern There was slight increase of peri-portal connective tissue

(f) Of the seven animals which received 30 *to* 49 *injections*, three showed a finely granular liver, while in four instances the surface of the organ showed a hobnail roughness (Plate XLI, figs 1 and 2)

Microscopically, the liver in the former group showed only irregular loose stellate areas of fibrosis (Plate XLII, fig 6), often containing pigmented mononuclear phagocytes, and either no loss of the parenchymal pattern, or only slight tendency to pseudo-lobulation In the latter group the capsule of the organ was slightly thickened and finely irregular , from the wedge-shaped areas of fibrous depressions proceeded loose fibrous tissue bands, which were continuous with the connective tissue network which divided the parenchymal mantle into numerous rounded islands of varying sizes (Plate XLII, figs 7 and 8) , the connective tissue network was loose and cellular but had undergone collagenous change (Plate XLII fig 8) and radiated mainly from the sclerosed hepatic veins , in its wider nodes, the connective tissue-network also enclosed the portal spaces The presence of pseudo-bile canaliculi in the connective network was not a marked feature The pseudo-lobulation was well marked , the pseudo-lobules occasionally showed sclerosed

and eccentric hepatic veins, the hepatic cords in the pseudo-lobules were hypertrophic and occasionally showed fatty degeneration Pigmented mononuclear phagocytes were found around the hepatic veins and occasionally around the portal spaces, in the connective tissue-network Silver impregnation of the reticulum showed a collagenous loose, connective tissue-network, well-marked pseudo-lobulation, a scarcity of the hepatic terminals and sclerosis of the bigger divisions of the hepatic venous tree (Plate XLII, fig 8), the reticulum in the pseudo-lobules was deficient (Plate XLII, fig 8) The portal venous tree showed no remarkable changes, beyond congestion The Kupffer cells showed fine, granular, brownish pigment in them

The hobnail appearance of the liver was more marked in the animals (Nos 10, 11 and 36) which received the drug also orally, for varying periods, in addition to the subcutaneous injections

(*g*) The appearances, macroscopic and microscopic of the liver in the animals (6 rabbits and 2 albino rats) which received the drug only *by mouth,* were similar to those seen in animals which received 2 to 6 injections subcutaneously [*vide (c)* above]

II   The spleen —The spleen in all the instances showed marked congestion and hæmosiderosis Reticulo-endothelial hyperplasia was occasionally seen

III   Other organs —The *lungs* showed sub-pleural hæmorrhages and marked congestion Catarrhal or suppurative bronchitis, bronchiolitis peri-bronchial fibrosis or broncho-pneumonia were occasionally found The *kidneys* showed congestion and occasionally moderate cloudy swelling of the convoluted tubular epithelium

Sub-serous hæmorrhages were commonly seen The *other organs* generally showed engorgement

Discussion

Attempts at inducing hepatic cirrhosis in experimental animals by repeated administraton of small doses of carbon tetrachloride have been productive of encouraging results A review of the subject is found in a paper by Moon (1934), central fibrosis of the liver without loss of lobular pattern, following carbon tetrachloride poisoning, was reported by Gardner and his associates (*loc cit*), cirrhosis of the liver with pseudo-lobulation was reported by Midorikawa (1925), Lamson and Wing (1926), Albot (1931) and others, but the type of cirrhosis was not emphasized, van der Scheuren (1932), Bollman and Mann (1931), Lacquet (1932), and others described the hepatic cirrhosis induced by chronic intoxication with carbon tetrachloride as having the characteristics of Lænnec's or atrophic cirrhosis On the other hand, Ramachandra Rao (1933) has emphasized that the distribution of fibrosis, in the hepatic cirrhosis induced by carbon tetrachloride intoxication, is mainly around the hepatic venous tree (unlike that seen in portal cirrhosis) and resembles that seen in a toxic cirrhosis

The significant findings in all the four instances, in this series, in which the liver showed hobnail roughness and marked cirrhosis were, as pointed out already, sclerosis of the bigger divisions of the hepatic venous tree a loose, cellular fibrous tissue-network, mainly around the hepatic venous tree, and marked

pseudo-lobulation   These appearances are very much similar to those described in toxic cirrhosis (Radhakrishna Rao, 1935*a*)

Lacquet (*loc cit*) considered that the cirrhosis produced by carbon tetra-chloride cannot be caused by necrosis of the hepatic parenchyma, as the fibrosis after repeated administration of the drug did not depend on the extent of the necrotic lesions of the parenchyma , repeated poisoning was said to exhaust the regenerating power of the liver and stimulate the mesenchyma to more active proliferation   But in preparations especially stained to show the reticulum of the liver by the silver impregnation method (Foot and Menard, *loc cit*), the genesis of fibrosis in the cirrhosis induced by carbon tetrachloride poisoning could be traced to a disorganiza-tion, collapse condensation and sclerosis of the reticulum of the sinusoidal capillary bed around the hepatic terminals consequent to the necrosis of the parenchyma in the same area , moreover, the looseness of the fibrous tissue and the absence of any inflammatory reaction at any stage of its development suggest that the cirrhosis, following chronic intoxication with carbon tetrachloride, is of the nature of a replacement fibrosis   As pointed out already the appearances in the liver are very suggestive of a toxic cirrhosis

The peri-central necrotic lesions in the early stages, in the animals in the present series, are similar to those described, both in experimental animals (Lacquet, *loc cit* , Ramachandra Rao, *loc cit* , Gardner and his associates, *loc cit* , etc) and in human cases (Keher and Onendal, quoted by McGuire, 1932) after carbon tetra-chloride poisoning   But unlike the human cases (McGuire), the kidney changes in the present series of animals were not marked

The intense engorgement of the viscera after the administration of carbon tetrachloride no doubt suggests that the drug has an action on the vascular system But it is difficult to explain the peri-central distribution of the necrotic lesions in the hepatic parenchyma   This peculiar zonal distribution of the parenchymal lesions has been attributed (Ramachandra Rao, *loc cit*) to a vasoconstriction and spasm of the sinusoidal capillaries of the hepatic venous end, due to an imbalance of the vasomotor mechanism of the liver   But the sinusoidal capillary congestion in the liver, following the administration of carbon tetrachloride in the present series of animals, was more or less uniform throughout the lobule and evidences of vaso-constriction in the central zones were not particularly striking   A direct hepato-toxic action of the drug, however, appears improbable on account of the peri-central distribution of the necrotic lesions in the hepatic parenchyma

A deficiency of the reticulum in the liver of the albino rat, unlike that seen in the human liver, probably explains its deficiency also in the pseudo-lobules in the cirrhotic livers of some of the animals of this series   In the albino rat, the reticular tissue is mainly found around the portal and hepatic venous trees, while the peri-sinusoidal reticulum, especially in the middle zones, is scanty

The absence of pseudo-bile canaliculi, both in the cirrhotic and non-cirrhotic livers in this series in which regenerative changes were marked, is additional evidence that these structures do not take part in the regeneration of hepatic tissue (Radha-krishna Rao, 1935*b*)

The results of this experimental study suggest that the sub-acute toxic necrosis and cirrhosis observed in the livers of children suffering from the so-called ' *infantile*

biliary cirrhosis ' (Radhakrishna Rao, 1934, 1935*b*) are similar to those seen in the experimental animal and are due to the slow, persistent and sub-acute action of a toxin, the exact nature of the toxin, however, is not clear from this study

TABLE

Showing details of the course of the experiment

Serial number	Experimental animal	CARBON TETRACHLORIDE		Number of days of experiment	REMARKS
		Subcutaneous injections	Oral administration (m = minims)		
1	Albino rat			193	Control—died after 193 days
2	,			105	Control—died after 105 days
3	,,	46		241	Liver—early cirrhosis
4	,	45		228	Liver—marked cirrhosis
5	,,	33		166	Liver—early cirrhosis
6		1		12 hours	Died 12 hours after injection
7	,	24		132	
8	.	12		72	
9	,	48		259	Liver—early cirrhosis
10	,	49	10 m daily for 146 days	447	Liver—marked cirrhosis
11	,,	48	10 m daily for 168 days	277	Liver—marked cirrhosis
12	,,	1			Died immediately after injection
13	Guinea pig	7		36	
14	,,	5		21	
15	,,	1		2	
16	,,	10		59	
17	,,	5		25	
18	,,	1		3	
19	,,	,		22	

TABLE—*concld*

| Serial number | Experimental animal | Carbon tetrachloride | | Number of days of experiment | REMARKS |
		Subcutaneous injections	Oral administration (m = minims)		
20	Guinea-pig	1		3	
21	,,	1		1	
22	,,	1		1	
23	,,				Control
24	,,				Control
25	Albino rat	16		108	
26	,,	7		47	
27	Guinea pig	4		23	
28	,,	4		21	
29	,,	3		15	
30	,,	2		11	
31	,,	2		10	
32	Albino rat	1		2	
33	,,	1		3	
34	,,	2		8	
35	,,	2		8	
36	,,	42	10 m daily for 91 days	265	Liver—marked cirrhosis
37	Rabbit		10 m daily	30	
38	,,		,,	,,	
39	,,		,,	,,	
40	,,		,,	,,	
41	,,		,,	,,	
42	,,		,,	,,	
43	Albino rat		,,	,,	
44			,,	,,	

SUMMARY

Repeated administration of small doses (0 1 c c to 0 2 c c once or twice weekly) of carbon tetrachloride subcutaneously to albino rats produced toxic cirrhosis The fibrous tissue was mainly distributed around the hepatic venous tree, which showed sclerotic changes in its larger divisions, its genesis in the cirrhosis thereby induced was traced to a disorganization, collapse, condensation and sclerosis of the peri-sinusoidal reticulum around the hepatic terminals, consequent on a necrosis of the parenchyma in the same area

ACKNOWLEDGMENTS

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THE STUDY OF EPIPHYSEAL UNION FOR DETERMINING THE AGE OF SOUTH INDIANS

A STUDY OF ONE HUNDRED CASES, CHIEFLY FROM THE MADRAS SCHOOLS AND COLLEGES, AGES RANGING FROM 10 TO 23

BY

M J S PILLAI, L R C P & S, ETC, D R (Edin),

Medical Superintendent, the Barnard Institute of Radiology, Government of Madras, Madras

As the study of epiphyses is of medico-legal importance, carefully selected cases with age certificates were alone taken for this purpose   To obtain the exact age of persons is a difficult matter in a country where illiteracy is common   Whereas other methods of fixing the age of a girl or a youth is a matter of opinion, in radiology we have definite data enabling us to get the age as accurately as possible

A study has been made of the age at which epiphyseal union takes place in one hundred persons between the ages of 10 and 23 years   The details of the findings are given in the Table   .

It will be seen that epiphyseal union of the South Indians takes place at ages earlier than those published in standard works, which appear to be the average for natives of temperate climates   The average age of fusion of the epiphyses has been found to be about two to three years in advance of the age incidence in temperate climates   It is noted that the epiphyseal union in guls is a little in advance of that of boys   It has also been found that the phalanges of fingers unite even earlier than the other epiphyses   In the same individual there are parts which show variations from the normal and this may be due to factors which hastened or retarded the epiphyseal union   It should be mentioned that in these examinations, special care was taken with regard to the technique of taking the radiographs, as otherwise distortions would have vitiated the results by altering the radiographic appearance

Cases of maldevelopment or arrest of growth in children due to various diseases in the early ages have been eliminated in this examination

In order to determine the age, more than one part which unites at the same age has to be taken, one confirming the other   For the purpose of this paper radiographs of elbow, wrist, phalanges, knee, ankle, foot and hip were taken, and films of the

skull were also made including the jaw, to note the development of pituitary fossa and teeth respectively   The pituitary condition occasionally reveals factors hastening or retarding the growth and fusion of epiphyses of the skeletal system

Our findings corroborate to a great extent the findings of Major Hepworth, I M S, whose study on this question is published in the *Indian Medical Gazette* of March 1929, page 128

My grateful thanks are due to Captain Barnard, Director of the Institute, for his generous help in providing all facilities for conducting this and other research work at the Institute

TABLE

Showing the age of ossification of the epiphyses of long bones of students of Madras and the comparative figures of European statistics

| Name of epiphyses | THE BARNARD INSTITUTE OF RADIOLOGY | | AVERAGE OF WESTERN STATISTICS | |
| | | | AGE OF FUSION | |
	Ages of fusion	REMARKS	Anatomy books	'Radiology', Holms and Ruggles
	Years		Years	Years
	(a) (b)			
Pisiform bone calcifies at	10 12		10	10
Phalanges of fingers	14 17	The terminal phalanges fuse first	20	18
Metacarpals	14 17		20	18
Ulna—				
Olecranon	14 16		Puberty	17
Distal end	14 18	The ulna fuses a little earlier than radius	21	18
Distal end of radius	14 18		21	20
Head of radius	14 17		20	17
Outer condyle of humerus	13 14		17	17
Inner condyle	14 17		18	18

Based on data collected in over 100 South Indians with authentic ages
Figures under (a) in column 1 represent the age of commencement of fusion
    ,,   ,,   (b) ,,   ,,   ,,   ,,   ,, ,, ,, completion of fusion

TABLE—*concld*

Name of epiphyses	The Barnard Institute of Radiology			Average of Western Statistics	
	Ages of fusion	Remarks		Age of fusion	
				Anatomy books	'Radiology', Holms and Ruggles
	Years			Years	Years
	(a) (b)				
Scapula—					
Corocoid process	13 14			Puberty	15
Acromian process appears {	13			Puberty	} 20
	14 18			20–25	
Head of humerus	14 17			20	18
Phalanges of toes	14 17			18	18
Metatarsals	14 17			18	18
Posterior epiphyses of oscalsis	14 17			18	18
Distal end of tibia	} 14 17			18–20 { tibia	18
„ „ „ fibula				fibula	20
Proximal end of tibia	} 14 17	Fibula unites later { than tibia		20 tibia	22
„ „ „ fibula				20–25 fibula	24
Distal end of femur	14 17			21	20
Head of femur	14 15			20	19
Greater trochanter {	14 17	} Lesser trochanter fuses earlier than { the greater one		19	18
	12			12 (appears)	13
Lesser trochanter appears {	14 17			18	17
Acetabulum	11 14			17	16
Crest of ileum appears {	13			Puberty (appears)	15
	14 18			20–25	23

Based on data collected in over 100 South Indians with authentic ages

Figures under (a) in column 1 represent the age of commencement of fusion

„ „ (b) „ „ „ „ „ „ „ completion of fusion

NOTICE.

The following has been received for publication —

—Editor

SECOND INTERNATIONAL CONGRESS FOR MICROBIOLOGY.

London, 25th July—1st August, 1936.

THE Congress will be officially opened on Saturday evening, the 25th July, 1936 This will be followed by official receptions by His Majesty's Government, by the Royal Society and other societies Excursions and visits to important institutes and laboratories have been arranged

Already 20 workers belonging to the Medical, Veterinary and Biological sciences have signified their desire to take part in the Congress Prospective members are requested to communicate, without delay, either with Dr A C Ukil, Secretary, Indian Committee of the International Society for Microbiology, All-India Institute of Hygiene and Public Health 21, Chittaranjan Avenue, Calcutta, or direct with Dr R St John-Brooks, Honorary General Secretary, Second International Congress for Microbiology, Lister Institute, Chelsea Bridge Road, London, S W 1, England, for a copy of the Registration Form and the Programme and to forward the membership fee of £ 1 sterling to Dr J T Duncan, Treasurer, Second International Congress for Microbiology, London School of Hygiene and Tropical Medicine, Keppel Street, London, W. C 1 Members should also intimate the Honorary General Secretary in London whether they would be accompanied by ladies

The headquarters of the Congress will be located at the University College, Gower Street, London, W C 1, where the scientific meetings will also be held Messrs Thos Cook & Sons, Ltd, of Berkeley Street, London, W 1, the American Express Co, Inc, of 6, Haymarket, London, S W 1, and Messrs Dean and Dawson Ltd, of 7, Blandford Square, London, N W 1, have been appointed official Travel Agents of the Congress Information as to special travel facilities, hotels in London, etc, may be obtained on application to these firms in London or at any one of their respective branches or agencies in Great Britain or abroad

In accordance with the statutes of the Society communications may be made to the Congress in English, French or German The Honorary General Secretary will be glad to hear from any prospective member who desires to take part in the general discussion on any of the prescribed subjects All openers of discussions and subsequent contributors must forward to the Honorary General Secretary, Dr R St John-Brooks, before 1st April, 1936, abstracts of their statements for incorporation in a special brochure available at the opening of the Congress These abstracts are strictly limited to 600 words in case of openers and to 300 words in case of

subsequent contributors   Openers will be limited to 20 minutes, invited contributors to 15 minutes and speakers in the general discussions to 10 minutes, at the President's discretion   It is hoped that interested workers of the Medical, Veterinary and Biological sciences will take advantage of this opportunity for making personal contacts between experts assembling from all over the World

PROVISIONAL PROGRAMME

Presidents of Honour—Past President Professor JULES BORDET (Brussels), Sir JOHN McFADYEAN (London), Sir ROBERT MUIR (Glasgow), Professor Geo   H   F   NUTTALL (Cambridge)

President—Professor J   C   G   LEDINGHAM, Lister Institute, London, S W 1

Presidents of Sections

Section	*President*
1 General Biology of Micro organisms	Prof E GOTSCHLICH (Heidelberg)
2 Viruses and Virus Diseases in Plants and Animals	Prof R DOERR (Basel)
3 Bacteria and Fungi in relation to Disease in Man, Animals and Plants	Dr E J BUTLER (London) and Prof H ZINNSER (Boston)
4 Economic Bacteriology (Soil, Dairying and Industrial)	Prof R E BUCHANAN (Iowa)
5 Medical, Veterinary and Agricultural Zoology and Parasitology	Prof E BRUMPT (Paris)
6 Serology and Immunochemistry	Prof K LANDSTEINER (New York)
7 Microbiological Chemistry	Prof A HARDEN (London)
8 Specific Immunization in the Control of Human and Animal Diseases	Prof W H PARK (New York)

The following is a list of the subjects for discussion in the different Sections —

Section 1.—GENERAL BIOLOGY OF MICRO ORGANISMS

Subjects for Discussion

Selective bacteriostasis   Inhibitory action on the growth of bacteria and fungi of (1) Substances of known constitution and (2) Products of the growth of micro organisms   Importance in the preparation of selective culture media

Nutritional factors associated with the growth of micro organisms   (*Joint with Section* 7)

Preservation of cultures of micro organisms   Latency   Methods of preservation of delicate organisms   Preservation of virulence and antigenic structure   Changes in the character of bacteria in culture media unfavourable for rapid growth

Life cycles of bacteria   Symbiotic associations   Filterable forms

Anærobic bacterial metabolism   (*Joint with Section* 7)

Bacterial photo synthesis   (*Joint with Section* 7)

Variation   Relation of changes in morphological and cultural characters to changes in chemical composition and to alterations in antigenic structure, toxin production and pathogenicity (*Joint with Sections* 3 and 6)

Section 2.—VIRUSES AND VIRUS DISEASES IN ANIMALS AND PLANTS

Subjects for Discussion

The general characteristics of viruses, including bacteriophage

Modes of transmission and paths of infection in virus diseases

Evidence concerning the agency of viruses in the aetiology of new growths

Mechanism of immunity in virus diseases and practical applications thereof

Section 3.—BACTERIA AND FUNGI IN RELATION TO DISEASE IN MAN, ANIMALS AND PLANTS

Subjects for Discussion

The significance of serological and cultural types of bacteria and fungi pathogenic to man, animals and plants in relation to epidemic, epizootic and epiphytic outbreaks of disease

Pathogenic streptococci Relation to scarlatina, puerperal fever, erysipelas, tonsillitis, acute rheumatism and infective endocarditis in man, and to mastitis, lymphangitis and suppurative conditions in animals

Mycoses in man, animals and plants Taxonomy Mechanism of pathogenic action Relation to saprophytic species and conditions of saprophytic growth

Bacteria causing acute inflammations of the alimentary tract and their mechanism of action (Amœbic infections included)

Pathogenic anærobic bacteria

Variation Relation of changes in morphological and cultural characters to changes in chemical composition and to alterations in antigenic structure, toxin production and pathogenicity (*Joint with Sections* 1 *and* 6)

Section 4.—ECONOMIC BACTERIOLOGY —SOIL, DAIRYING AND INDUSTRIAL MICROBIOLOGY

Sub section   I   Dairy Microbiology

Sub section   II   Industrial Microbiology

Sub section III   Soil Microbiology

Subjects for Discussion

(Sub section I) Significance and estimation of the numbers and types of bacteria in milk, including thermoduric and thermophilic organisms The need for adopting uniform methods

(Sub section II) The microbiology of water supplies

(Sub section II) (*a*) The microbiology of perishable fresh foods, other than milk and milk products (*b*) The microbiology of canned foods, other than milk and milk products

(Sub section III) The physiology of nitrogen fixing organisms and the biochemistry of nitrogen fixation

(Sub section I) Factors determining the behaviour of micro organisms in milk and milk products

(Sub sections II and III) (*a*) The process of decomposition of plant remains in soil, manure and compost heaps (*b*) The microbiology of ensilage production

(Sub section II) The destruction and protection of woods and cellulosic materials

(Sub section II) Problems of biochemical purification of sewage and of trade effluents

(Sub section II) Recent advances in fermentation industries

(Sub section III) Economic importance of the autotrophic bacteria

(Whole Section—Sub sections I, II and III) Yeast metabolism (*Joint with Section* 7)

Section 5.—MEDICAL, VETERINARY AND AGRICULTURAL ZOOLOGY AND PARASITOLOGY

Subjects for Discussion

Resistance of animal parasites to conditions external to the host

Factors which influence the transmission of infections by arthropod vectors

Typhus fever and the Rickettsias

The biology of the malarial parasites of man and animals

Coccidia in relation to domesticated animals

Subjects for Discourses

Chemotherapy—mechanism of drug action and drug resistance

The parasitic nematodes of plants

Immunity against animal parasites

Section 6.—SEROLOGY AND IMMUNOCHEMISTRY

Subjects for Discussion

The structure of natural and synthetic antigens   (*Joint with Section 7*)

Immunity reactions in relation to antigenic structure and variation in bacteria

Principles and methods for the quantitative determination of antigens and antibodies, including their diagnostic application

Blood groups and organ specificity

The significance of allergy in disease

Variation   Relation of changes in morphological and cultural characters to changes in chemical composition and to alterations in antigenic structure, toxin production and pathogenicity (*Joint with Sections 1 and 3*)

Section 7.—MICROBIOLOGICAL CHEMISTRY

Subjects for Discussion

The structure of natural and synthetic antigens   (*Joint with Section 6*)

Nutritional factors associated with the growth of micro organisms   (*Joint with Section 1*)

Metabolic products of the lower fungi

Intermediate carbohydrate metabolism of micro organisms

Influence of substrate on the chemical potentialities of the cell

Anaerobic bacterial metabolism   (*Joint with Section 1*)

Bacterial photosynthesis   (*Joint with Section 1*)

Yeast metabolism   (*Joint with Section 1*)

Section 8.—SPECIFIC IMMUNIZATION IN THE CONTROL OF HUMAN AND ANIMAL DISEASES

Subjects for Discussion

The control of diphtheria and whooping cough by means of specific immunizing reagents

The prophylaxis and serum treatment of human and animal diseases caused by anaerobic bacteria

The prophylaxis and serum treatment of pneumonia

The relative value of anti toxic and anti bacterial immunity in the prophylaxis and treatment of human and animal diseases in which the invasion by the causative bacterium may occur in a focal or generalized form

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